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Studies on Yeast Metabolism

5. THE TREHALOSE CONTENT OF BAKER'S YEAST DURING ANAEROBIC FERMENTATION*

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(*Received* 10 *March* 1955)

In a previous study of the synthesis of cellular carbohydrate during fermentation by D.C.L. baker's yeast (Trevelyan, Gammon, Wiggins & Harrison, 1952) little variation was found in the trichloroacetic acid-soluble carbohydrate, of which the main constituent is trehalose (Trevelyan & Harrison, 1952). The low concentration of glucose in the medium used, about 5 mM, was thought to have restricted trehalose synthesis. The present paper reports measurements of trehalose in yeast during fermentation of high concentrations of sugar. As Brandt (1941) reported that trehalose disappeared rapidly from the yeast cell as a result of growth in a suitable medium, but increased in amount during metabolism in the absence of assimilable nitrogen, particular attention has been paid to the effect on yeast trehalose of nitrogenous constituents of the medium. Disappearance of trehalose during budding has been noted also by Pollock & Holmstrom (1951). Recently a study of some aspects of the connexion between carbohydrate and nitrogen assimilation in *Torulopsis utilis* has been published by Yemm & Folkes (1954).

METHODS

Fermentation was carried out at 30°, using, unless otherwise stated, a suspension of 2·5 g. (wet wt.) of pressed D.C.L. baker's yeast/80 ml. This was stirred by a stream of 150 ml. of N_2/min. Four separate batches of yeast (referred to below as yeast I, II, III or IV) were used in the investigation; one (yeast IV) had a much higher trehalose content than the rest. Fermentation was initiated by adding 20 ml. of sugar solution. After allowing 2 min. for mixing, the first sample was withdrawn for carbohydrate determination.

Media. The 'full medium' contained 0·05 M sodium succinate buffer, pH 5·5, 2 mM-KH_2PO_4, 8 mM-KCl, 5 mM-$MgSO_4$, 0·01 M-$(NH_4)_2SO_4$, 0·02 M DL-asparagine and 5 μM thiamine. The 'N-free medium' had no ammonium salts or asparagine. The 'P-free medium' contained 0·01 M-KCl, but no KH_2PO_4. The 'asparagine-free medium' had 0·05 M-NH_4Cl as sole nitrogen source. The fermentable sugars used were glucose, fructose or mannose, in an initial concentration of 0·3 M (unless stated otherwise) and sucrose or maltose, 0·15 M. In the most rapid fermentation the glucose concentration at the end of a 2 hr. experimental period was still enough to saturate the yeast (0·15 M).

Determination of carbohydrate. Trevelyan & Harrison (1952) described a rapid method for the routine determination of yeast carbohydrates based on the application of the anthrone technique either directly to the whole cells or to extracts containing principally trehalose, glycogen, mannan or glucan. The principles of this method were followed in the present work. Colour was developed, however, by the heat-of-mixing technique of Morris (1948). To determine TCA (trichloroacetic acid)-soluble carbohydrate (trehalose), 2 ml. of yeast suspension were pipetted into 3 ml. of ice-cold water: the cells were separated by centrifuging and washed twice with 5 ml. of cold water. The washed cells were then extracted three times with 4 ml. 0·5 M TCA, each extraction taking 20 min. The combined supernatants were diluted to 50 ml., and the carbohydrate content was measured by the anthrone method.

TCA-insoluble carbohydrate, which includes the polysaccharides glycogen, mannan and glucan (Trevelyan & Harrison, 1952), was determined by applying the anthrone technique directly to a suspension in water of the TCA-extracted cells. As it was thought that some loss of cellular carbohydrate might occur during washing of yeast with water before TCA extraction, a few determinations of TCA-insoluble carbohydrate were made in which the yeast sample was delivered directly into TCA to destroy instantaneously all enzyme action. Results were essentially unaltered, so this procedure was abandoned, as trehalose could not be determined on the same yeast sample.

Results are given as μmoles glucose/g. original wt. of yeast.

Fermentation rate. This was determined, in separate experiments, by absorption of CO_2 in alkali and titration of the carbonate after precipitation with barium ions (Trevelyan & Harrison, 1954*a*).

RESULTS

Identification of the TCA-soluble carbohydrate as trehalose

Resting yeast had been previously examined (Trevelyan & Harrison, 1952), but it seemed possible that fermentation, particularly of sugars other than glucose, might lead to an alteration in the nature as well as amount of the TCA-soluble carbohydrate. To test this, yeast after fermentation for 2 hr. in an N-free medium, of glucose, fructose, mannose or

* Part 4: Trevelyan & Harrison (1954*b*).

maltose, was washed and then extracted with 1 ml. of M TCA/g. yeast. Carbohydrates in the extract, before and after hydrolysis for 2 hr. at 100° in N-HCl, were identified by paper chromatography (Partridge, 1948). After development in a pyridine–butanol–water (20:30:13, v/v) solvent each extract gave a spot with an R_F value characteristic of trehalose, which reacted weakly with the silver nitrate reagent of Trevelyan, Procter & Harrison (1950). After hydrolysis this was replaced by a rapidly reacting spot with the R_F value characteristic of glucose. Glucose was identified in the hydrolysates also after development of chromatograms in a phenol–*iso*propanol–water (70:5:25, v/v) solvent, which gave a better separation of glucose and fructose. All chromatograms showed faint spots with R_F values smaller than that of trehalose, and the number and intensity of these were much increased in an extract of a yeast which had fermented glucose in a medium containing assimilable nitrogen. As these spots reacted with ninhydrin they were probably due to amino acids. However, as a further check, a heavily loaded chromatogram was cut into strips, and material on the paper was dissolved in water and tested with the anthrone reagent. Only from the trehalose region was anthrone-positive material obtained.

Trehalose breakdown and resynthesis during fermentation of glucose

In a preliminary experiment a high concentration of yeast (10%, w/v) was used, so that extracellular glucose was exhausted after 80 min. The medium contained glucose (0·2 M), but none of the salts or other constituents listed under Methods. Fermentation, for the first 40 min., was accompanied by a partial breakdown of yeast trehalose (Fig. 1). The subsequent resynthesis was interrupted by the exhaustion of fermentable substrate at 80 min., after which the level of trehalose was fairly stable. (Spiegelman & Nozawa (1945) have reported that yeast will not metabolize its intracellular carbohydrate in the absence of fermentable substrate.) During the initial period of trehalose breakdown the fermentation rate of the yeast increased (Fig. 1), and minimum content of trehalose coincided with the attainment of maximum fermentation rate.

Inhibition of trehalose resynthesis during assimilation of nitrogen by yeast fermenting glucose

That trehalose metabolism is not, however, linked in a simple manner with the fermentation rate was shown by comparing trehalose measurements made on yeast fermenting glucose in the different media described under Methods. These supported quite different fermentation rates, though in each case an initial period of rapidly rising fermentation rate could be distinguished. After this initial phase the rate remained constant at 55 μmoles CO_2/min./g. yeast in thiamine-free medium. After fermentation for 35 min. in N-free medium the rate had reached its maximum (69 μmoles of CO_2/min./g.), after which time it declined by about 10% per hour. In P-free medium (at 20 min.) the rate was 61 μmoles of CO_2/min./g. The rate continued to increase; the acceleration, however, rapidly died away to give

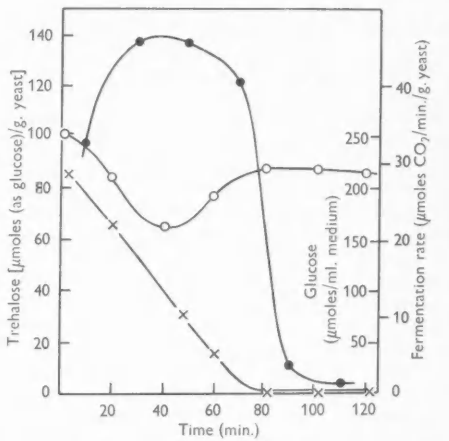

Fig. 1. Trehalose breakdown and resynthesis during fermentation of glucose by 10% (w/v) yeast II. Medium, 0·2 M glucose, no other additions. ○, yeast trehalose; ●, fermentation rate; ×, extracellular glucose.

a rate of only 74 μmoles of CO_2/min./g. after 120 min. of fermentation. Enzyme synthesis occurring in full medium supported a linear increase of rate from 77 μmoles of CO_2/min./g. at 20 min. to 114 at 120 min. As may be seen from Figs. 2 and 3, yeast trehalose broke down in the initial stage of fermentation, and to a greater extent than when a simple solution of glucose had been used as the fermentation medium (cf. Fig. 1). Appreciable resynthesis was observed only when assimilable nitrogen was omitted from the medium. In thiamine-free medium the fermentation rate was lower than in N-free medium, but only a slow resynthesis of trehalose was observed; in full medium the rate was much higher, but again resynthesis was inhibited. The substitution of casein hydrolysate for asparagine and ammonium sulphate was without effect (Fig. 3). By comparing the composition of yeast after fermentation of glucose for 2 hr. in (*a*) full medium and (*b*) N-free medium, it was found that the incorporation (mainly as protein) into the acid-insoluble fraction of 1 μmole nitrogen

reduced the amount of glucose assimilated as trehalose and polysaccharides by about 0·5 μmole. This corresponds to the replacement of carbohydrate formation by synthesis of an equal weight of protein.

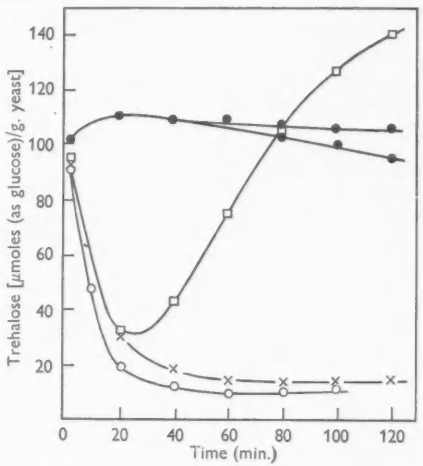

Fig. 2. Effect of constituents of the medium on trehalose breakdown and resynthesis during fermentation by yeast I. ○, Full medium + glucose (0·3 M); ×, P-free medium + glucose (0·3 M); □, N-free medium + glucose (0·3 M); ● (upper curve), N-free medium + maltose (0·15 M); ○ (lower curve), full medium + maltose (0·15 M).

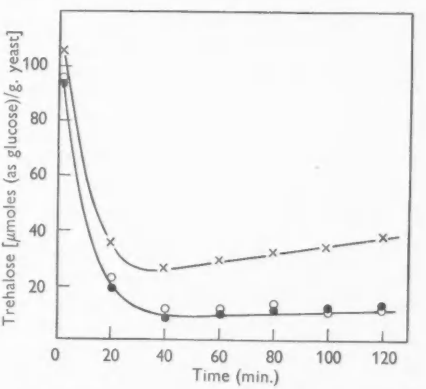

Fig. 3. Effect of constituents of the medium on trehalose breakdown and resynthesis during fermentation of glucose (0·3 M) by yeast III (cf. Fig. 2). ○, Full medium; ●, N-free medium + amino acids (casein hydrolysate, 0·05 M in terms of total N, +0·5 mM L-tryptophan); ×, thiamine-free medium.

Stability of trehalose in the absence of fermentable substrate

Yeast trehalose was stable when incubated with ammonium sulphate (asparagine-free medium) in the absence of glucose. Seven determinations of trehalose content, made at 20 min. intervals, gave the mean value of 143 μmoles (as glucose)/g., with an S.D. of ±5. This experiment provided incidentally a fair test of the reproducibility of the analytical procedure.

Relation between the initial breakdown of trehalose and the metabolism of intracellular nitrogen reserves

It is natural to inquire whether nitrogenous constituents of the yeast itself are responsible for the initial breakdown of trehalose, which occurred

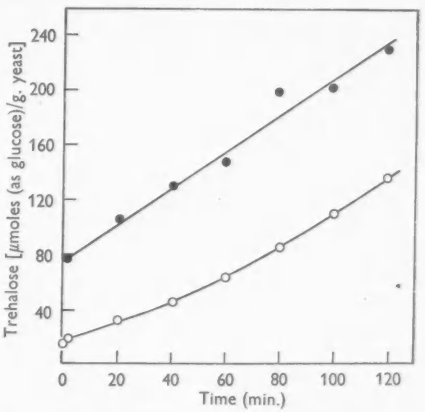

Fig. 4. Elimination of the initial phase of trehalose breakdown during fermentation in N-free medium (0·3 M glucose), by pre-treating yeast IV for 60 min. with glucose in full medium (○), or for 80 min. with glucose in N-free medium (●). After pre-treatment the yeast was washed and transferred to fresh N-free medium.

even in N-free medium. As washing the yeast with 40 times its bulk of N-free medium before fermentation did not eliminate trehalose breakdown (Fig. 6), contamination of the yeast by extracellular nitrogen compounds was ruled out. Yeast, however, contains intracellular amino acids; the composition and metabolism of this nitrogen reserve have been considered by Halvorson & Spiegelman (1953). Partial depletion of the amino acid reserve was brought about by fermentation in N-free medium, as shown by a decrease in the intensity of spots on a paper chromatogram of TCA extracts prepared as described under Methods and sprayed with ninhydrin. After fermentation for 80 min., when the

trehalose level of the yeast had returned to its initial value, the yeast was separated by centrifuging and washed free of medium. A second sample of yeast, fermented for 60 min. in full medium, retained its nitrogen reserves. When glucose in N-free medium was added to both samples of yeast, the trehalose level immediately began to increase (Fig. 4). The difference between the rates of synthesis in the two cases was not sufficient to support the idea of a relation between trehalose breakdown during fermentation by untreated yeast and the metabolism of intracellular amino acid reserves.

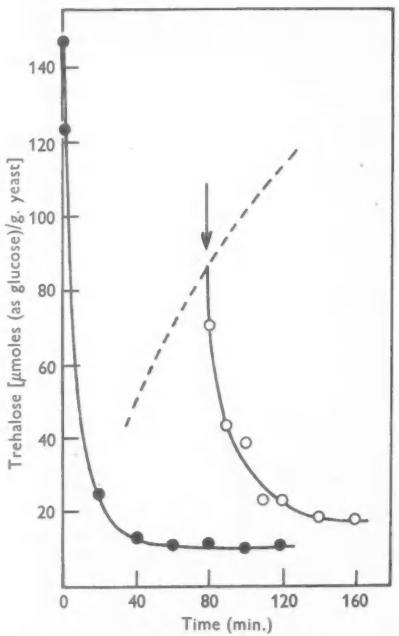

Fig. 5. Interruption of trehalose resynthesis (yeast IV) by addition of 0·05 M-NH_4Cl (↓) after fermentation of 0·3 M glucose had proceeded for 80 min. in N-free medium (○): for comparison, the progress of trehalose resynthesis during fermentation in N-free medium is plotted (dotted line). ●, Failure of mM uridine or adenine (identical results) to promote trehalose resynthesis during fermentation of 0·3 M glucose in full medium.

It will be recalled that the initial period of trehalose breakdown was also one of increasing fermentation rate. Treatment of the yeast with glucose in the manner described abolished not only trehalose breakdown, but also the lag in attaining the maximum rate of fermentation (Trevelyan & Harrison, 1954*a*).

Further experiments on the effects of nitrogen assimilation on trehalose metabolism

After fermentation of glucose for 80 min. in an N-free medium, the yeast had resynthesized approximately the amount of trehalose lost in the initial breakdown period. Ammonium chloride added at this point immediately caused the level of yeast trehalose to decline once more (Fig. 5).

It was thought possible that competition between the reactions of trehalose and protein synthesis for common coenzymes, such as the adenine nucleotides or uridine diphosphoglucose [reported by Leloir & Cabib (1953) to be involved in the synthesis of trehalose], might explain the antagonistic effects of nitrogen assimilation on trehalose synthesis. Uridine or adenine, possible precursors of coenzymes, were therefore added to yeast fermenting glucose in full medium, but no effect was observed (Fig. 5). In this experiment only, trehalose was determined immediately before the addition of glucose as well as 2 min. afterwards. Even in 2 min. the breakdown of yeast trehalose is appreciable (Fig. 5).

Trehalose levels during fermentation of maltose

Maltose was fermented much more slowly than glucose, and the rate increased with time owing to synthesis of the adaptive enzyme maltase. Only minor changes in yeast trehalose accompanied maltose fermentation (Fig. 2), though a slow breakdown of trehalose in the second hour was induced by addition to the medium of assimilable nitrogen.

Trehalose levels during fermentation in N-free medium of sucrose, fructose, or mannose

Glucose could be replaced by sucrose, fructose, or mannose without significant alteration in fermentation rates. In each case (Fig. 6) an initial period of trehalose breakdown was succeeded by resynthesis. With fructose or mannose, differences were recorded between the minimum trehalose level and the rates of resynthesis, as compared with those characteristic of glucose fermentation.

Effect of azide

It was shown previously (Trevelyan *et al.* 1952) that above a certain concentration sodium azide induced a breakdown in yeast trehalose during fermentation of glucose. As Fales (1953) obtained different results, possibly owing to his using a different type of yeast, the effect of azide on trehalose metabolism was re-investigated. Previous results were confirmed: as Fig. 7 shows, mM-NaN_3 caused the breakdown of trehalose and of part of the TCA-insoluble carbohydrate (polysaccharides) when added to yeast fermenting glucose in N-free

medium either 2 min. before sugar addition, or after fermentation had proceeded for 60 min. (This concentration of azide inhibited fermentation somewhat, the respective rates being 40 and 50 μmoles of CO_2/min./g. as compared with the control rate of over 60.) Azide action on trehalose metabolism is particularly interesting, since this inhibitor has been shown to affect enzyme (protein) synthesis by yeast (Spiegelman & Reiner, 1947).

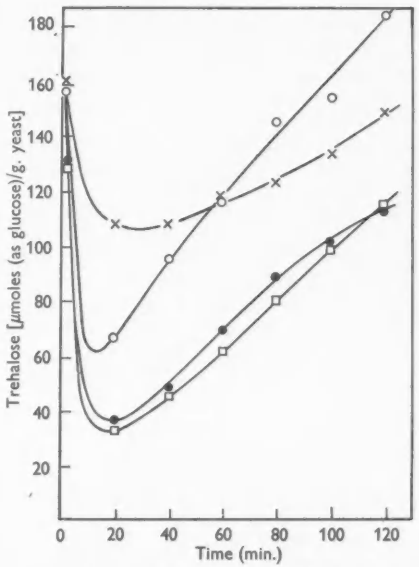

Fig. 6. Trehalose content of yeast IV during fermentation, in N-free medium, of ○, fructose; ●, glucose; ×, mannose; □, sucrose. Before the glucose fermentation, the yeast was washed to ensure that possible extracellular contaminants had no influence on the initial trehalose breakdown.

Level of TCA-insoluble carbohydrate

The changes of yeast trehalose level during the fermentation of glucose were accompanied by similar, though less striking, variations in TCA-insoluble carbohydrates (Table 1). Later experiments (unpublished) have shown these changes to be due largely to alterations in glycogen content.

DISCUSSION

The enzyme systems involved in the synthesis and degradation in the yeast cell of trehalose (or indeed of any other yeast carbohydrate) have not been worked out in detail. The overall kinetics, e.g. of

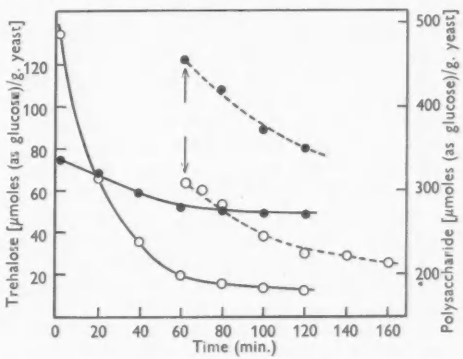

Fig. 7. Breakdown in yeast IV of trehalose (○) and TCA-insoluble carbohydrate (including glycogen) (●), in the presence of mM sodium azide. Full line: azide added 2 min. before glucose. Dotted line: azide added 60 min. after glucose (↕).

Table 1. *Synthesis of yeast TCA-insoluble carbohydrate during fermentation of glucose (G), maltose (MA) or mannose (M)*

(*a*) marks estimations in which yeast was delivered directly into trichloroacetic acid (see Methods).

Yeast (no.)	I	I	I	I	III	III	III	IV
Sugar fermented	G	G	G	MA	G	G	G	M
Medium	Full	P-free	N-free	N-free	Full	Thiamine-free	Full, casein hydrolysate as N source	N-free
Time (min.)	TCA-insoluble carbohydrate [μmole (as glucose)/g. yeast]							
	(*a*)	(*a*)						
2	344	341	310	344	309	320	309	378
20	325	341	335	346	282	311	286	393
40	348	371	416	358	309	338	284	413
60	355	390	494	373	317	359	308	480
80	363	409	529	401	338	371	348	528
100	393	431	580	431	352	389	361	585
120	428	468	593	455	398	416	385	645

trehalose metabolism, involve not only the kinetics and equilibrium conditions of these systems, largely unknown, but also the concentrations of intermediates such as glucose 6-phosphate which are maintained at a steady level in the cell as a result of the complex interaction of several or all of the enzyme systems of the Embden–Meyerhof–Parnas scheme.

The formation of trehalose 6-phosphate during fermentation by dried yeast or cell-free yeast extracts (Myrbäck, 1949) suggests that this substance plays an important role. Recently (P. F. E. Mann & J. S. Harrison, 1955, private communication) an improved version of the ion-exchange technique used by Trevelyan, Mann & Harrison (1954) has been applied to show that trehalose 6-phosphate is formed in the yeast cell within 10 min. of adding glucose. A simple explanation of the stability of trehalose in the absence of fermentable substrate would be to postulate an initial phosphorylation to trehalose 6-phosphate as the initial step in the breakdown of this carbohydrate. This type of explanation is difficult to apply to the case of glycogen, which, as we have seen, behaves similarly to trehalose during fermentation.

Nitrogen assimilation involves the formation of amino acids, principally from intermediates formed by reactions branching off the main pathway of fermentation at the pyruvate level. The reactions of glycogen, and probably trehalose synthesis (Myrbäck, 1949), diverge at the glucose 6-phosphate level. It is at first sight difficult to see how these two groups of synthetic reactions can interact. That an indirect effect of nitrogen assimilation on carbohydrate formation is, in principle, possible is shown by considering a property of the ammonium ion not directly related to its function as a source of assimilable nitrogen. Muntz (1947) reported that NH_4^+ (and K^+) stimulated phosphofructokinase in yeast-maceration juice. The effect of such stimulation, occurring during fermentation *in vivo*, would be either to increase the rate of fermentation or, if the rate is restricted by reactions other than the phosphofructokinase system, to lower the steady-state level of fructose 6-phosphate and indirectly of glucose 6-phosphate. Conditions would then be less favourable to glycogen or trehalose synthesis.

Phosphofructokinase is linked to hexokinase through a common coenzyme, adenosine triphosphate (ATP). Theoretically, the steady-state level of glucose 6-phosphate during fermentation should be regulated not so much by the absolute amount of phosphofructokinase, as by its amount relative to hexokinase. This latter enzyme has different reaction rates and Michaelis constants towards glucose, fructose and mannose (Slein, Cori & Cori, 1950), conceivably accounting for the differences in trehalose levels found during fermentation of the different sugars.

Since a period of adaptation to the medium eliminates the initial phase of trehalose breakdown when glucose is added to the yeast in a nitrogen-free medium, it is suggested that ionic exchanges between the yeast and the medium are involved in the transition from conditions favouring trehalose breakdown to conditions favouring synthesis during the first 30 min. of glucose fermentation. Alterations in the internal concentration of ions such as K^+, Mg^{2+}, $H_2PO_4^-$ may, through effects on hexokinase and phosphofructokinase, and possibly other reactions of alcoholic fermentation, alter the steady-state levels of glucose 6-phosphate and those of other intermediates important in carbohydrate assimilation.

Since both carbohydrate and nitrogen assimilation are processes which utilize the adenosine-triphosphate (ATP) generated during fermentation, the inhibition of carbohydrate reserve formation during (anaerobic) nitrogen assimilation may be regarded as a mechanism ensuring the distribution of the limited amount of ATP available for synthetic reactions in the most biologically effective manner.

SUMMARY

1. Fermentation of glucose, sucrose, fructose or mannose by baker's yeast is accompanied by an initial breakdown in yeast trehalose.

2. In nitrogen-free medium this initial breakdown is succeeded by trehalose resynthesis. Assimilable nitrogen prevents resynthesis during fermentation of glucose for 2 hr.

3. After fermenting glucose with or without assimilable nitrogen yeast transferred to fresh nitrogen-free medium starts synthesizing trehalose immediately glucose is added.

4. Yeast trehalose shows only minor changes during fermentation of maltose.

5. The breakdown of yeast trehalose during glucose fermentation in presence of mM azide has been confirmed.

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Studies on Yeast Metabolism

6. EFFECTS OF THIAMINE ON NITROGEN ASSIMILATION DURING FERMENTATION, AND ON FERMENTATION KINETICS

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Trevelyan & Harrison (1954*a*, *b*) showed that the addition of thiamine brought about a rapid lowering in the steady-state concentration of pyruvate in the yeast cell during fermentation, accompanied by only a slight immediate increase in fermentation rate. They suggested that carboxylase could therefore be considered to be one of the factors limiting fermentation rate only when thiamine was omitted from the medium, and that some other enzyme system or systems limited the fermentation rate of thiamine-saturated yeast.

Thiamine, in an appropriate medium, is known to promote an increase in fermentation rate which continues for several hours. This long-term action of thiamine, which was discovered by Atkin, Schultz & Frey (see their review in Anderson, 1946), is considered in the present paper in relation to the short-term effect on pyruvate levels mentioned above.

While this work was in progress, a paper by Maesen (1953) appeared in which it was reported that the long-term increase in fermentation rate mediated by thiamine was accompanied by a proportionate increase in the carboxylase activity of the yeast cells (determined after disruption by freezing and thawing). Rate increases were observed only when assimilable nitrogen (ammonium sulphate) was supplied to the yeast, but the rate of its incorporation into yeast protein was unaffected by thiamine. Thiamine thus appeared to have a specific effect on carboxylase synthesis, and this enzyme system was, Maesen concluded, the limiting factor of fermentation under his experimental conditions.

METHODS

Fermentation technique

Yeast was taken as required from a master sample of D.C.L. pressed baker's yeast stored in the refrigerator over a period of not more than 3 weeks. When necessary, the various master samples are distinguished by the letters A, B etc.

Full medium M1 contained sodium succinate buffer, pH 5·5 (0·05 M succinic acid partly neutralized with 75 mM-NaOH), 0·01 M-KH_2PO_4, 0·01 M-$(NH_4)_2SO_4$, 0·02 M DL-asparagine, 2·5 mM-$MgSO_4$, mM-$ZnSO_4$, mM-$CaCl_2$ and 0·1 μmole thiamine/g. yeast. Medium M2 contained sodium succinate buffer, ammonium sulphate and asparagine in identical concentration: the other constituents were 5 mM-$MgSO_4$, 2 mM-KH_2PO_4, 8 mM-KCl and 0·2 μmole thiamine/g. yeast. Essentially similar rates were obtained during fermentation for 2 hr. with the two media. Thiamine-free medium, N-free medium, P-free medium contained all constituents except thiamine, ammonium sulphate and asparagine, or phosphate, respectively. Asparagine-free medium had 25 mM-$(NH_4)_2SO_4$ as sole nitrogen source.

Fermentation was carried out at 30°, the suspension being stirred by bubbling N_2 through at about 150 ml./min. Variation of the gas flow between 100 and 200 ml./min. had no effect on the fermentation rate. Unless stated otherwise, the fermentable sugar was 0·3 M glucose. Usually the final volume of 100 ml. contained 2·5 g. (fresh wt.) of yeast, which had a dry-matter content of 27%. In previous studies of the short-term effect of thiamine, double this concentration of yeast was used (Trevelyan & Harrison, 1954*b*). Although the fermentation rate/g. of yeast was the same during the first 40 min. of fermentation, if 2·5 g. or 5 g. of yeast were used, it was found that a steady increase in rate after this time was observed only at the lower level of yeast concentration.

Fermentation rate, as an average over 10 or (normally) 20 min. intervals, was determined after absorption of CO_2 in alkali (Trevelyan & Harrison, 1954*a*).

Analytical methods

Results of estimations of yeast constituents are stated as μmoles/g. *original wt. of yeast*, measuring changes in the whole culture, not in the individual cell.

Phosphate uptake. This was followed by the Gomori method (Gomori, 1942) applied to the supernatant after centrifuging off the cells. Preliminary experiments established that no phosphorus could be detected in the medium after exhaustion of orthophosphate.

Ammonia uptake. Diffusion into boric acid, using the Conway microdiffusion unit (Conway, 1947), was employed.

Cell counts. Triplicate counts, each of some 500 cells, were made with a haemocytometer.

Nucleic acid. Nucleic acids were determined in the cells, after three 20 min. extractions with chilled trichloroacetic acid (TCA), by the technique of Schneider (1945). This has been previously applied to yeast (DiCarlo & Schultz, 1948), though some doubt has been cast on the suitability of the method (Bourdet, Mandel & Guillemet, 1951).

Metaphosphate. 'Acid-insoluble' metaphosphate was calculated from the acid-labile phosphorus in the Schneider extract, as described by Wiame (1949).

Acid-soluble ribose. This was expected to give a rough idea of the level of coenzymes in the cell and was determined in the TCA extract of yeast (washed free from medium), using the method of Mejbaum (1939). Trehalose interfered, but after fermentation for 40 min. in N-containing media the trehalose level was low enough (Trevelyan & Harrison, 1956) for the ribose estimation to give reliable values.

Acid-insoluble nitrogen (AIN). After extraction by cold TCA the suspension, containing 50 mg. yeast, was centrifuged, and the cells were washed with water and transferred, using the minimum amount of water, to a Pyrex tube for Kjeldahl digestion according to the directions of Koch & McMeekin (1924).

Intracellular pyruvate. This was estimated as previously described (Trevelyan & Harrison, 1954*a*).

Intracellular amino acids. Yeast cells were centrifuged, washed twice by centrifuging with ice-cold water, and the drained cells were extracted by shaking for 30 min. at room temperature with 1 ml. of M TCA/g. of yeast. Paper chromatography of the supernatant was carried out by using the solvent and ninhydrin reagent described by Toennies & Kolb (1951); other solvents were butanol–acetic acid–water (100:30:20, v/v, top layer), and pyridine–butanol–water (100:150:65, v/v). As the amount of water remaining in the packed cells after centrifuging was unknown, spot intensities were compared with a standard prepared by extracting a duplicate sample of yeast with TCA to which had been added 200 μmoles of alanine. The comparison was made after cutting out spots from the paper and dissolving the coloured material in 75% (v/v) acetone, a solvent proposed by Naftalin (1948). Optical density at 600 mμ. of the solutions was then measured.

RESULTS

Effect on fermentation rate of constituents of the medium

The first 40 min. of glucose fermentation by D.C.L. yeast may be considered as a lag period. In all media examined, fermentation rate rises during this period. Yeast which has been adapted to the medium by a preliminary fermentation and which has been centrifuged and washed will begin fermentation when transferred to fresh medium without an induction period. After this time, fermentation rate may steadily increase, remain constant, or decline, according to the composition of the medium.

A steady increase in rate requires a source of assimilable nitrogen (Maesen, 1953). The necessity for adding to the medium other substances, such as thiamine or phosphate, appears to depend upon the type of yeast used, as may be seen by comparing the results of this paper (Figs. 1–4) with those of

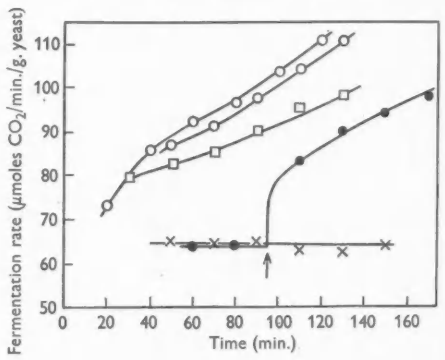

Fig. 1. Fermentation rate of yeast A in medium M1. ○, Full medium; upper curve, fresh yeast; lower curve, yeast stored for 13 days at 4°. □, Asparagine-free medium; ×, thiamine-free medium; ●, thiamine added after fermentation for 95 min. in thiamine-free medium (↑).

Maesen (1953) and Atkin *et al.* (cited in Anderson, 1946). Schultz, Atkin & Frey (1937) and Heyns (1939) found that thiamine could be replaced by 4-amino-5-ethoxymethyl-2-methylpyrimidine, the yeast being apparently capable of synthesizing the thiazole portion of the thiamine molecule. With D.C.L. yeast, on the other hand, thiamine (5 μM) could be replaced by high concentrations (100 μM) of 5-β-hydroxyethyl-4-methylthiazole: the pyrimidine part of the thiamine molecule (5 or 100 μM) was less effective than thiamine itself in promoting rate increases, as was the thiazole at the lower concentration.

In N-free medium, the fermentation rate (after the lag period) decreased with time. After addition of ammonium sulphate and asparagine at 107·5 min. the rate showed only a small immediate increase, after which the rate/time curve ran parallel to that given by yeast fermenting from the beginning in

full medium (Fig. 2). Supplementing full medium with nicotinamide resulted in a greater acceleration: adenosine, on the other hand, had the opposite effect (Fig. 2). Schultz, Atkin & Frey (1938) reported a smaller stimulatory effect of nicotinamide and found that adenylic acid was without effect.

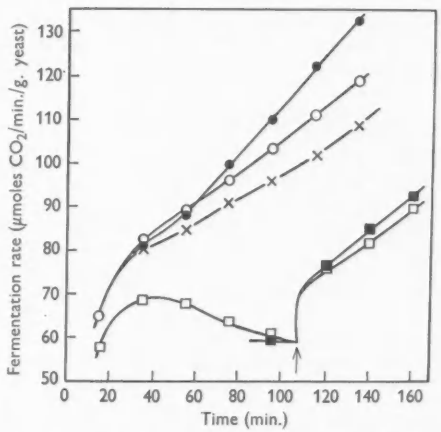

Fig. 2. Fermentation rate of yeast E in medium M2. ○, Full medium; ●, full medium + nicotinamide; ×, full medium + adenosine; ■, thiamine-free medium for 107·5 min., then thiamine added (↑); □, N-free medium for 107·5 min., then ammonium sulphate + asparagine added (↑). The rates in asparagine-free medium + nicotinamide are not plotted: they were close to those in full medium unsupplemented with nicotinamide.

Yeast which had been pretreated with glucose in full medium, washed, and then transferred to N-free medium showed a greater decline in fermentation rate than untreated yeast, or yeast which had been pretreated in N-free medium (Fig. 3). Addition of ammonium salts to the medium did not restore the rate to the initial value.

The increase in fermentation rate induced by thiamine and assimilable nitrogen was not maintained unless phosphate was also present in the medium: in P-free medium the rate appeared to approach a limiting value (Fig. 4). Restoration of phosphate caused the rate to rise immediately to that of a control fermentation in full medium. As the first sample of yeast tested had an anomalously low fermentation rate, this observation was repeated on a fresh batch of yeast.

Glucose could be replaced by fructose, in full medium, without change in the fermentation rate/time curve (Fig. 5). Mannose was fermented slightly more slowly and the general shape of the curve was altered (Fig. 5).

In thiamine-free medium, a constant fermentation rate was observed after the initial induction period. When, after 95 min. fermentation in thiamine-free medium, thiamine was added, the rate showed only a small immediate increase, after which the rate/time curve ran parallel to, but below, that for fermentation in full medium (Figs. 1, 2).

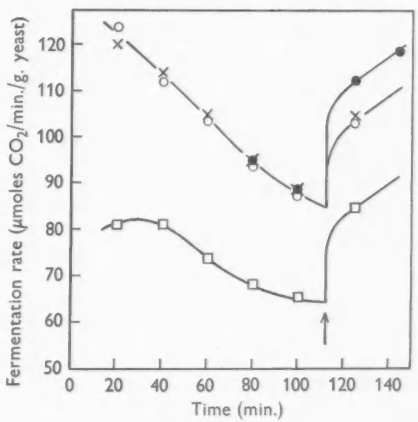

Fig. 3. Fermentation rates in N-free medium. □, Yeast G prefermented for 2 hr. in thiamine-free medium, thiamine added before fermentation in N-free medium (↑); ○, ●, yeast prefermented in full medium; ×, yeast prefermented in full medium + nicotinamide. In ●, 0·02 M-NH_4Cl + 0·1 M glucose added at 112·5 min.; in ○, ×, and □, NH_4Cl only (↑).

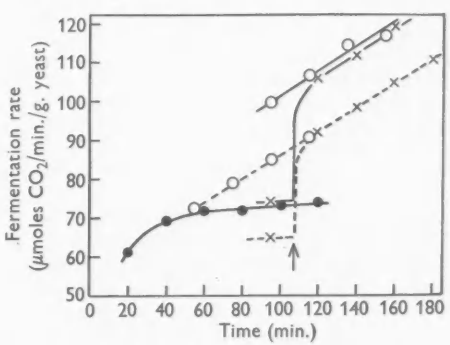

Fig. 4. Effect of phosphate on fermentation rate. ●, P-free medium, yeast C; ○, full medium: full line, yeast E; broken line, yeast D; ×, phosphate added after fermentation in P-free medium for 107·5 min. (↑).

A large number of experiments were made in which thiamine was added after fermentation for 107·5 min. in thiamine-free medium supplemented with (*a*) casein hydrolysate, or single amino acids; (*b*) alkaline or acid-hydrolysed ribonucleic acid, purines, pyrimidines, adenosine or uridine; (*c*) vitamins, singly or in combination; (*d*) formate, acetate or butyrate, as sources of 1- and 2-carbon

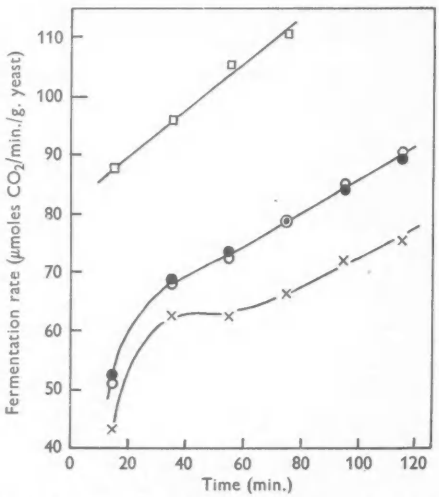

Fig. 5. Fermentation rate (yeast D, medium M2) of ○, glucose, ●, fructose, and ×, mannose. □, Mannose fermentation by yeast after prefermentation for 130 min. with glucose.

units used in synthetic processes. Rates were measured (i) at 95 min., to record the constant rate in thiamine-free medium, (ii) at 120 min., to determine the rapid increase in rate on addition of thiamine, and (iii) at 140 min., to determine, in conjunction with the 120 min. value, the rate of increase in full medium. The additions had little effect. Some of the rates recorded, in the order (i), (ii), (iii) above, were: control, 59, 76, 85 μmoles of CO_2/min./g.; most effective amino acids were 5 mM L-glutamine with rates of 63, 84, 92; and 0·01 M DL-serine with rates of 54, 76, 90; inhibitory amino acids were 0·01 M DL-cysteine with rates of 52, 65, 70 and 0·01 M DL-methionine with rates of 62, 68, 72. Of group (*b*) adenine and adenosine were most active. These compounds slightly increased the rate in thiamine-free medium and also the immediate effect of thiamine, but decreased the subsequent increase in fermentation rate. For example, mM adenine had rates of 56, 79, 85. Vitamins had little effect. In full medium the rates at comparable times were 103, 112, 121. When air (150 ml./min.) was passed through the fermentation vessel in place of nitrogen, the rates altered only slightly, to 104, 116, 125 respectively. A greater effect was shown on the rate in thiamine-free medium, and on the effect of restoring thiamine, typical figures being 69, 91, 101. Supplementation of the medium with casein hydrolysate, etc., again produced only minor effects.

Other (anaerobic) experiments showed (*a*) that destruction of added thiamine was not involved, since increasing the thiamine addition by a factor of 20 was without effect, and (*b*) that inhibitory

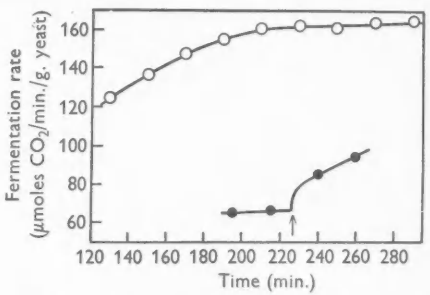

Fig. 6. Fermentation rate, 1·25% (w/v) yeast F in medium M2 containing 0·45 M glucose. ○, Full medium; ●, thiamine added after fermentation for 227·5 min. in thiamine-free medium (↑).

substances released into the medium during thiamine-free fermentation were not important, since after centrifuging and washing the yeast, and adding thiamine immediately on starting fermentation in fresh medium, the rate was still well below that in the full medium. In another experiment the yeast concentration was reduced to 1·25 g./100 ml., and the time of fermentation extended to over 4 hr. After 195 min. in thiamine-free medium the rate was 66 μmoles of CO_2/min./g., and the same rate was recorded at 215 min. Thiamine was added at 227·5 min.; at 240 min. the rate was 85 μmoles of CO_2/min./g., rising to 94 at 260 min. In full medium the rates at these times were over 160 μmoles of CO_2/min./g. (Fig. 6).

Effect of thiamine on the level of intracellular pyruvate

The fermentation rate of D.C.L. baker's yeast has been shown to be related to the intracellular pyruvate concentration (Trevelyan & Harrison, 1954*a*). This relation is described by an equation of the Michaelis–Menten type, with a K_m of 3 μmoles of pyruvate/g. of yeast. From the fermentation rate and intracellular pyruvate level may be calculated the fermentation rate at infinite pyruvate

concentration, which, it has been suggested, may be a measure of carboxylase activity *in vivo*. This theoretical maximum rate is increased by the addition of thiamine.

After fermenting glucose in thiamine-free medium for 100 min., the yeast was separated by centrifuging, washed, and a second fermentation under the same conditions was set up. After 5 min. the intracellular pyruvate reached the high level of 15·1 μmoles/g. of yeast. Excess of thiamine was added at 7·5 min., so that the total period of thiamine-free fermentation was the same as in the experiments discussed in the preceding section. The pyruvate level fell rapidly to 2·5 μmoles/g. at 10 min., 2·5 at 15 min., and 2·4 at 20 min. By extrapolation the fermentation rate immediately after addition of thiamine was found to be 79 μmoles of CO_2/min./g. The calculated rate at infinite pyruvate concentration was 170 μmoles of CO_2/min./g. after addition of thiamine. For the yeast before fermentation in thiamine-free medium it was found to be 160 μmoles of CO_2/min./g.

When the yeast fermented glucose in full medium, however, quite different results were obtained. Yeast thus treated was separated after 210 min. and transferred to thiamine-free medium. Its initial fermentation rate was 182 μmoles of CO_2/min./g., which was unaltered by further addition of thiamine, which had, moreover, little effect on the intracellular pyruvate. This was 4·85 μmoles/g. (original wt. of yeast) at 15 and 20 min. (thiamine-free fermentation) and at 30 min. after thiamine addition the level of pyruvate was 4·47. Since both cell counts and optical density of the yeast suspension showed that the actual amount of yeast had doubled during the 210 min. fermentation, the level of pyruvate/g. actual wt. of yeast during the second fermentation was about 2·5 μmoles/g., and the fermentation rate 90 μmoles of CO_2/min./g. That is, during fermentation in full medium, the calculated maximum fermentation rate increased in parallel with the observed fermentation rate, so that the intracellular pyruvate concentration remained constant.

If the calculated maximum rate is equated with carboxylase activity, the results agree with those of Maesen (1953): that carboxylase is synthesized in proportion to the increase in fermentation rate, and that fermentation in the presence of thiamine increases the rate of synthesis. But, during fermentation in full medium, the pyruvate level is not high enough to support the contention that the carboxylase system is rate-limiting.

Effect of thiamine on some synthetic activities of yeast during fermentation

Results summarized below indicate that thiamine increased the extent of several general synthetic processes, though its effect was not as marked as on fermentation rate.

Cell division. The mean rate of cell division during fermentation for 2 hr. in full medium was 13 ± 3 %/hr. The deoxyribonucleic acid content of the suspension increased by 12 %/hr., but by only 4 % in the absence of thiamine. After 4 hr. fermentations, microscopic examination showed obviously greater numbers of buds, and more frequent chains of cells, when thiamine had been added: the increase in cell count was approximately doubled by thiamine.

Nitrogen and phosphorus metabolism. D.C.L. yeast contained 1220 μmoles of acid-insoluble nitrogen (AIN)/g. About 200 μmoles were present as ribonucleic acid (RNA), corresponding to 50 μmoles of RNA phosphorus. In full medium, AIN increased by 266 μmoles/g. of yeast/hr., but by only 136 in thiamine-free medium. Omission of phosphate from the medium considerably lowered incorporation of nitrogen into AIN to 134 with, and 94 without, thiamine. Using the high-phosphate medium M1 (see Methods), thiamine was shown to increase the uptake of phosphate (Fig. 7). Most of the phosphate taken

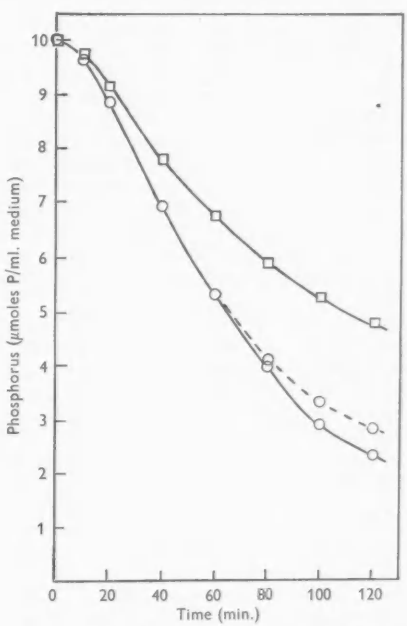

Fig. 7. Effect of thiamine on phosphate uptake by yeast B from medium M1. □, Thiamine-free medium, with or without asparagine; ○, broken line, asparagine-free medium; full line, full medium.

up appeared in the hot TCA extract; after 2 hr. in full medium 285 μmoles of phosphate were taken up, the total phosphorus in the hot TCA extract increased from 52 to 226, and metaphosphate from 3 to 117 μmoles P/g. In full medium the rate of RNA formation was 21·7 μmoles of P/g./hr. and was reduced to 13·6 by omission of thiamine. With ammonium sulphate as the sole source of nitrogen the rates of formation with and without thiamine were 16·6 and 11·1 μmoles of P/g./hr. respectively. Uptake of ammonia during fermentation in medium M 1 was increased by thiamine from 215 to 298 μ-moles/g./hr. In view of the effect of thiamine on nucleic acid formation it is interesting to note that no effect on the level of acid-soluble ribose during fermentation for 2 hr. could be shown.

Amino acid metabolism

If addition of thiamine during fermentation were actually to stimulate overall protein synthesis, its effect could conceivably be either (*a*) direct, by virtue of some coenzyme requirement for amino acid synthesis or subsequent incorporation into protein, or (*b*) indirect, due to its marked influence on the intracellular pyruvate level, when one might expect an effect on the amino acid directly derived from pyruvate, namely alanine.

It was found that after fermentation of glucose for 4 hr. in full medium, the only amino acids detectable in the medium by paper chromatography [phenol–*iso*propanol–water (70:5:20, v/v) solvent] were asparagine and aspartic acid. When thiamine was omitted from the medium, however, an amino acid with the R_F of alanine appeared. This excretion of alanine was evidence of a high intracellular level during thiamine-free fermentation. The examination of instantaneous intracellular levels during fermentation, necessitating rapid destruction of enzyme action (e.g. by TCA), was not attempted in view of the difficulty of separating amino acids heavily contaminated by constituents of the medium. The pattern of amino acids extracted after washing the yeast free from medium, however, clearly showed the expected influence of thiamine. Fortunately, after paper chromatography alanine was well separated from the other amino acids present in TCA extracts of yeast. Detailed identification of the other amino acids was not attempted: according to Halvorson & Spiegelman (1952, 1953*a*, *b*) glutamic acid and lysine are important constituents. With D.C.L. yeast, it was found that about one-quarter of the colour produced after spraying chromatograms with ninhydrin was due to alanine. Gale (1953) states that the amino acid generally present in highest concentration within bacterial cells is alanine.

After fermentation in N-free medium for 2 hr. of glucose, fructose or mannose the level of intracellular amino acids decreased: maltose fermentation under the same conditions scarcely affected the amino acid pattern. Addition to the medium of ammonium sulphate counteracted the disappearance of amino acids during fermentation of glucose; the alanine spot on the chromatograms after ninhydrin treatment was slightly less intense than that given by the original yeast. Asparagine addition produced minor intensity differences, and caused

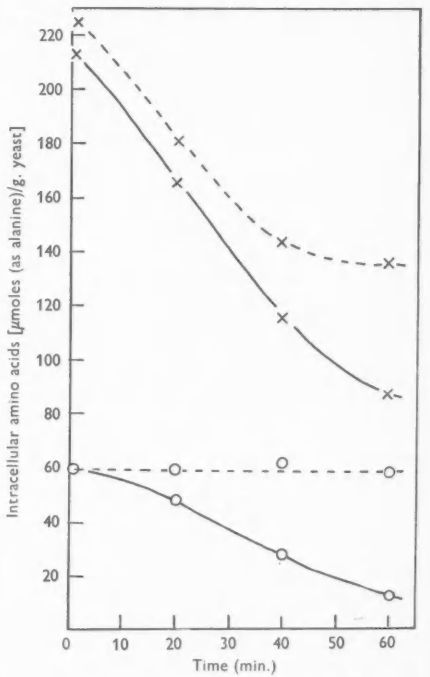

Fig. 8. Effect of thiamine on utilization of intracellular amino acids during fermentation in N-free medium. After applying ninhydrin to a paper chromatogram developed in butanol–acetic acid, the blue colour was eluted and compared with an alanine standard. ○, Alanine spot; ×, total amino acids; broken line, thiamine absent; full line, thiamine present.

the appearance of a new spot which reacted orange-brown with ninhydrin and was presumably due to asparagine itself. Omission of thiamine during fermentation in the presence of ammonium sulphate increased the intensity of spots due to minor components of the amino acid pattern, and most noticeably increased the amount of alanine present to 3·3 times that in either the original yeast or yeast which had fermented glucose in the presence of thiamine and ammonium sulphate.

Thiamine also influenced the utilization of intracellular amino acids during the first hour of glucose fermentation in N-free medium (Fig. 8). With thiamine, the intensity of the alanine spot rapidly diminished, whereas no net change occurred in the absence of thiamine. In the latter case, the intracellular pyruvate level has been shown to rise steadily during the first 40 min. or so of the fermentation (Trevelyan & Harrison, 1954*a*), thus increasingly favouring alanine formation.

DISCUSSION

It is clear from the results that baker's yeast, when fermenting glucose in a suitable medium, is capable of anaerobic growth for at least 3 hr. Growth includes cell division and also the formation of fermentation enzymes and coenzymes, the result of which is a steady increase in fermentation rate. The effect on fermentation rate of ammonium salts or asparagine is not immediate, but manifests itself gradually as the incorporation of nitrogen into cell substance proceeds. The effect of thiamine, which is also time-dependent, is therefore presumably on some synthetic process included in the term 'growth'. Reduction in the rate of protein synthesis when thiamine is omitted from the fermentation medium, it is suggested, is due to the interference with the incorporation of amino acids into protein by the high intracellular concentration of pyruvate or alanine. The magnitude of this interference may depend upon the thiamine present in the particular yeast culture used, since Maesen (1953) found no effect of additional thiamine on overall protein synthesis.

Moreover, this reduction in protein synthesis appears too small to explain the much more marked effect on fermentation rate increase and on carboxylase synthesis, whether the course of this process is followed by enzyme assay (Maesen, 1953) or by determination of intracellular pyruvate concentration. The pyruvate measurements suggest that factors other than carboxylase are involved.

The phosphate requirement again may depend on the amount present in the yeast culture. With D.C.L. yeast, which has a relatively low phosphate content, fermentation rate does not increase steadily unless the medium is supplemented with phosphate. Unlike assimilable nitrogen or thiamine, phosphate has a large immediate effect on fermentation rate. This is probably due to an increase in the intracellular concentration of phosphorylated substrates of the fermentation enzymes. It should be pointed out that, when the cells of a yeast suspension divide, the effectiveness of a fixed amount of any substrate or dissociable coenzyme decreases, since it is the intracellular concentration which is kinetically relevant. Any cell division occurring in P-free medium would therefore tend to decrease the fermentation rate, and the shape of the rate/time curve (Fig. 4) reflects the opposition of some such factor to the effect of enzyme synthesis or other processes tending to increase fermentation rate.

As the substrate of carboxylase is non-phosphorylated, it would appear unlikely that this enzyme is the limiting factor during fermentation by D.C.L. yeast in phosphate-free medium.

Only in the case of fermentation in thiamine-free medium does carboxylase appear to be rate-limiting. The constant fermentation rate observed, though the cells are slowly dividing, argues for rate control by an enzyme rather than by a coenzyme or by substrate levels.

In general, rate limitation by a single enzyme system of the Embden–Meyerhof–Parnas scheme is likely to be found only in yeast nutritionally deficient in one or more factors. The reason for this is that such limitation implies a very high intracellular level of one particular substrate, a level which moreover changes considerably with a slight alteration in the overall rate of metabolism. The possibility of inhibition of other cellular activities is clear. Yeast fermenting in full medium maintains a constant intracellular pyruvate concentration about sufficient to half-saturate the carboxylase system.

The decrease of fermentation rate with time when yeast ferments in the absence of assimilable nitrogen also implies that the amount of enzyme cannot be the sole rate-determining factor in fermentation.

SUMMARY

1. Acceleration of the fermentation rate of baker's yeast by thiamine depends on synthesis of fermentation enzymes from nitrogenous constituents of the medium.

2. This synthesis occurs in phosphate-free medium, but its effect on rate is masked by the dilution of intracellular phosphorylated substrates due to cell division.

3. This synthesis is restricted in the absence of thiamine. Cell division, nucleic acid and overall protein synthesis are less affected. The high intracellular level of pyruvate and alanine may interfere with protein synthesis.

4. Carboxylase synthesis during fermentation occurs only in the presence of thiamine, but the low intracellular pyruvate level shows that carboxylase is not a limiting enzyme in fermentation by thiamine-saturated yeast.

5. Fermentation-rate control appears to be a complex process involving factors other than the level of fermentation enzymes.

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The Effect of Certain Vitamin Deficiencies on Glycine Biosynthesis

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Conclusive proof that glycine is a dispensable amino acid has been obtained in recent nutritional experiments with synthetic diets (Rose, Burr & Sallach, 1952), but it is noteworthy that, at least in the rat, the amount of glycine synthesized by the growing animal exceeds its probable requirements by only a small margin (Arnstein & Neuberger, 1953*b*). Since glycine is a precursor of many important nitrogenous compounds such as creatine, purines and porphyrins (for a recent review see Arnstein, 1954), any significant decrease in the ability of the animal to synthesize this amino acid would be expected to have a profound influence on general metabolism and growth. It was therefore of interest to study the effect of vitamin deficiencies on glycine biosynthesis, and the present paper records experiments with guinea pigs deficient in ascorbic acid and rats deficient in pteroylglutamic acid and vitamin B_{12}. Only pteroylglutamic acid deficiency was found to affect the rate of glycine biosynthesis to a significant extent.

Serine is an important precursor of glycine (Shemin, 1946), but an alternative mechanism of glycine synthesis from glyoxylic acid or a closely related two-carbon acid has also been demonstrated (Weinhouse & Friedmann, 1951; Weissbach & Sprinson, 1953). Since pteroylglutamic acid, or more probably its tetrahydro derivative (Blakley, 1954), is required for the metabolic interconversion of serine and glycine (Elwyn & Sprinson, 1950), the decrease in glycine formation observed in pteroylglutamic acid deficiency may be used to assess the quantitative importance of serine as a glycine precursor.

Ascorbic acid increases the *in vitro* conversion of pteroylglutamic acid into N^5-formyltetrahydropteroylglutamic acid (citrovorum factor) (Nichol, 1953), possibly by facilitating the reductive step to tetrahydropteroylglutamic acid. This observation suggested that ascorbic acid deficiency might affect glycine biosynthesis indirectly and thus account for the marked decrease in muscle glycine of scorbutic guinea pigs (Christensen & Lynch, 1948). The present experiments show, however, that scorbutic guinea pigs are able to synthesize normal amounts of glycine.

Earlier experiments on the biosynthesis of choline from [α-^{14}C]glycine by the rat indicated a possible requirement of vitamin B_{12} for the formation of serine from glycine (Arnstein & Neuberger, 1952; Stekol, Weiss & Weiss, 1952). This possibility appeared to be supported by the subsequent observation that the conversion of glycine into the

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cysteine and glutamic acid moieties of glutathione is reduced in vitamin B_{12} deficiency (Anderson & Stekol, 1953). In more direct experiments it was found, however, that vitamin B_{12} does not affect the incorporation of [α-^{14}C]glycine into serine (Arnstein & Neuberger, 1953*b*), and we now report further experiments which show that in the rat neither the serine–glycine reaction nor the catabolism of [α-^{14}C]glycine is decreased by a deficiency of vitamin B_{12}.

EXPERIMENTAL

Animals and diets

Food consumption and body weights. The food consumption of the animals used in Expts. nos. 1–14 was measured daily; the rats were weighed every 2 or 3 days and the guinea pigs every 5 days. In the other experiments with guinea pigs (Tables 5 and 6) the animals were weighed every 5 days where indicated, but the food consumption was not determined.

Pteroylglutamic acid deficiency. Albino rats of the National Institute for Medical Research (Institute) strain were placed on the casein diet described below soon after weaning. After varying periods (see Table 1) the animals were fed on the synthetic amino acid diet AAD 3. Finally the same diet containing 0·1% of [^{15}N]glycine was fed for 14–20 days as shown in Table 1 (period III). In Expts. nos. 5–7 the development of pteroylglutamic acid deficiency as indicated by reduced growth (Fig. 1) was checked by leucocyte counts on the 16th, 29th and 38th day of the experiment. On the 29th day the leucocyte counts of the normal and deficient rats were 9900, 9600 and 5060, 6200, 4910 and on the 38th day 13120, 8300 and 2980, 4500 and 8400 respectively, the third deficient animal (Expt. no. 7) having been given pteroylglutamic acid during period III.

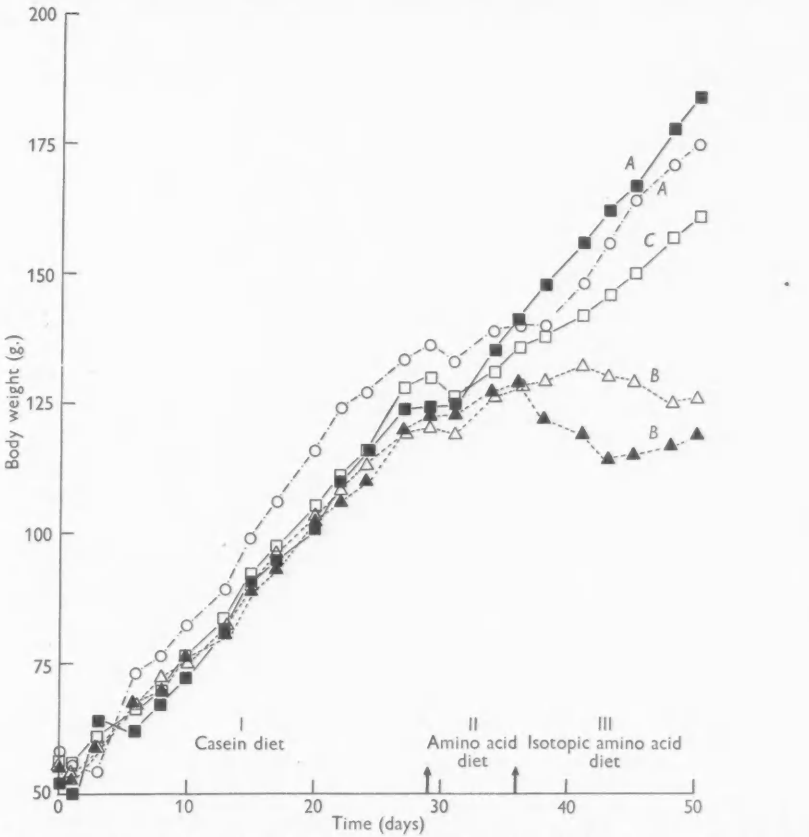

Fig. 1. Growth of normal and pteroylglutamic acid-deficient rats on casein and synthetic amino acid diets. *A*, normal animals; *B*, pteroylglutamic acid-deficient animals; *C*, deficient animal fed on normal diet during period III. The arrows designate change of experimental diets.

Vitamin B_{12} deficiency. Albino rats of Institute strain were used. In Expts. nos. 11 and 12 the animals were the same as those used in a previous investigation (Expts. nos. 18 and 19, Arnstein & Neuberger, 1953*a*). In the other experiments (Table 1, nos. 3, 3*a* and 4) the conditions were essentially the same as with the pteroylglutamic acid-deficient animals above, except that vitamin B_{12} was omitted from the diet. One group of rats (Expt. no. 4) was fed on diets deficient in both pteroylglutamic acid and vitamin B_{12}.

Ascorbic acid deficiency. Young male albino guinea pigs of Institute stock were fed on diet S or diet 41 for 2–3 weeks, the control animals being given 5 mg. of ascorbic acid/day. In a few experiments a second control group was fed on diet 18 with cabbage. After about 2 weeks the scorbutic guinea pigs began to lose weight. Some of the control animals also lost weight, but at *post mortem* all the deficient animals and none of the controls showed typical heavy haemorrhage, particularly around the joints and in the muscles of the hind legs.

Casein diet. This diet had the following composition (g./kg. of diet): casein ('vitamin-free', British Drug Houses Ltd.) 120; cane sugar, 360; maize starch, 390; cod-liver oil, 16; arachis oil, 64; salt mixture (Glaxo Laboratories, no. DL6), 40; succinylsulphathiazole, 10. In addition, 0·2% of DL-threonine, 0·3% of L-cystine, 0·25% of choline chloride and the following vitamins (mg./kg. of diet) were added: α-tocopherol, 40; thiamine, 10; riboflavin, 10; pyridoxine, 10; nicotinic acid, 10; *p*-aminobenzoic acid, 10; calcium D-pantothenate, 50; inositol, 100; biotin, 0·1; pteroylglutamic acid, 2, and vitamin B_{12}, 0·5. The last two vitamins were omitted in the deficient groups.

Diet AAD 3. This synthetic amino acid diet was identical with diet AAD 2 (Arnstein & Neuberger, 1953*a*) except for the following changes: L-glutamic acid was increased from 24 to 34 g., aspartic acid, proline and [α-^{14}C]glycine were omitted and homocystine was replaced by DL-methionine (10 g.). In Expts. nos. 5–10 glucose was used instead of cane sugar. During period II (see Table 1) the above diet was supplemented with non-isotopic L-alanine (5·9 g./kg.) (except in Expts. nos. 8 and 9, in which no alanine was fed) and glycine (1 g./kg.). The isotopic amino acid diet used in period III had the same composition, but the glycine was labelled with ^{15}N (15·76 atom % excess in Expts. nos. 1 and 2–4; 15·08 in Expts. nos. 1*a* and 10; 16·29 in Expts. nos. 8 and 9; 32·65 in Expts. nos. 5–7), and in some experiments the alanine and glucose were labelled with ^{14}C in connexion with another investigation (Arnstein & Keglević, 1956).

Diet 41. The composition was as follows (g./100 g. of diet): wholemeal flour, 46; Sussex ground oats, 40; fish meal, 8; dried skimmed milk, 3; cod-liver oil, 1; dried yeast, 1; NaCl, 1. For Expts. nos. 13 and 14, powdered diet 41 (1·5 kg.) was mixed with an aqueous solution of [α-^{14}C]glycine (1·4763 g.; specific radioactivity, 13·0 μc/g.) and the diet was then dried in an oven at about 40°. A portion of the diet was hydrolysed with 6N-HCl (about 10 vol.) for 18 hr. and glycine was re-isolated by using a Dowex 50 column and eluting the amino acids with HCl (1·5 and 2·5N). The specific radioactivity of the isolated glycine was 1·94 μc/g. and hence the glycine content of the diet (including the added [α-^{14}C]glycine) was 1·33%.

Diet 18. This diet had the following composition (g./100 g. of diet): grass meal, 30; bran, 15; groundnut cake, 15; linseed cake, 10; barley meal, 20; meat and bone meal, 8; $CaCO_3$, 1; NaCl, 1.

Diet S. This diet is the scorbutigenic diet described by Harris & Ray (1932).

Isolation of amino acids, hippuric acid and formate

Serine, glycine and alanine. The animals were killed under anaesthesia and the preliminary separation of amino acids from the mixed internal organs (liver, kidney, spleen, reproductive organs, heart and lungs) of each group which had received the same diet was carried out on a column of Zeo-Karb 225 cation-exchange resin (Permutit Co. Ltd.) as described previously (Arnstein & Neuberger, 1953*b*). In Expt. no. 1, serine and glycine were isolated as the *N*-benzoyl derivatives by method A of Arnstein & Neuberger (1953*b*), but the following modification was found more convenient and was adopted in subsequent experiments. Fractions containing serine and threonine or glycine and alanine from the ion-exchange column were evaporated to dryness and excess of HCl was removed *in vacuo*. The amino acid hydrochlorides were then converted into the free amino acids by absorption on Zeo-Karb 215 resin (Permutit Co. Ltd.) (approx. 5 g. of resin/100 mg. of hydrochlorides), the column was washed with water till the effluent became neutral and the amino acids were eluted with 0·2M-NH_3. The eluate was concentrated *in vacuo* almost to dryness, applied to Whatman no. 3 MM paper (load approx. 1 mg./cm.) and chromatographed for about 18 hr., using phenol–water (5:2, v/v) in the presence of NH_3. The position of the amino acids was detected by spraying narrow strips, cut out from the middle and both ends of the paper, with a solution of ninhydrin (0·2% in butanol). Serine, glycine or alanine was then eluted from the appropriate portions of the paper with water. The eluate was evaporated to dryness and the amino acids were crystallized from water by addition of ethanol. Their purity was checked by paper chromatography and by constant radioactivity on recrystallization.

Glutamic and aspartic acid. Fractions from the Zeo-Karb 225 column containing only glutamic acid or aspartic acid were concentrated to dryness *in vacuo* and excess of HCl was removed by drying the residue thoroughly in a vacuum desiccator over NaOH. The amino acid hydrochloride was dissolved in the minimum amount of water, the pH was adjusted to approx. 4–5 with pyridine, and ethanol was added to complete the crystallization of the amino acid. It was recrystallized from aqueous ethanol and the purity was checked by paper chromatography, as above, and constant radioactivity on further recrystallization from aqueous ethanol.

Hippuric acid. In the experiments described in Tables 5 and 6 the guinea pigs were kept in metabolism cages and urine was collected, usually for 24 hr. The filtered urine was acidified to Congo red with 2N-HCl, extracted with chloroform (0·05 vol.) and three times with ethyl acetate (1–2 vol.). The ethyl acetate extract was washed with a little water, dried (Na_2SO_4) and evaporated to dryness *in vacuo*. The solid residue was dissolved in the minimum amount of hot water, charcoal was added if necessary and the solution was filtered. Hippuric acid crystallized on cooling and was recrystallized (water), m.p. 191°.

Formate. Formate was isolated from the urine, oxidized to CO_2 and counted as $BaCO_3$, following the procedure of Weinhouse & Friedmann (1952).

Isotope estimations

Determination of ^{15}N. Samples containing not less than 1 mg. N were digested by the micro-Kjeldahl procedure. The NH_3 was distilled, absorbed in 0·02 N-HCl (5 ml.) and converted into N_2 with alkaline hypobromite (Bentley, 1952; Sprinson & Rittenberg, 1949). Excess of ^{15}N was estimated in a 60° mass spectrometer.

Radioactivity measurements. Samples of 'infinite thickness' were mounted on 1 cm.^{2} polythene disks (Popják, 1950) and counted with a helium-filled bell-shaped Geiger–Müller counter. The combined errors due to variations in sample preparation and statistical counting errors were about ±5%.

Labelled compounds

Uniformly labelled L-[^{14}C]serine and [α-^{14}C]glycine were purchased from the Radiochemical Centre, Amersham, and [^{15}N]glycine was prepared from potassium [^{15}N]phthalimide (Schoenheimer & Ratner, 1939).

all normal animals showed reasonably good weight-increases which were better than those of the animals fed on deficient diets. Vitamin B_{12} deficiency had little effect on growth when the amino acid diet contained methionine (Expts. nos. 3, 3*a* and 4), but deficient animals on a diet containing homocystine instead of methionine (Expt. no. 12) grew at a definitely suboptimal rate.

Effect of vitamin deficiencies on glycine metabolism

Pteroylglutamic acid deficiency. The incorporation of ^{15}N from dietary [^{15}N]glycine into serine was very much reduced in pteroylglutamic acid-deficient rats (Table 2). This is clearly shown by a comparison of the isotope content of the isolated glycine and serine as given by the glycine/serine

Table 1. *Food consumption and growth of rats on normal, pteroylglutamic acid-deficient and vitamin B_{12}-deficient diets*

For details of animals and diets see Experimental section. The food consumption and body weights are the combined individual values of animals in each group.

Expt. no.	No. and sex of rats M.	F.	Diet	Experimental periods (days) I Casein diet	II Amino acid diet	III Isotopic amino acid diet	Amount of isotopic diet eaten (g.)	Mean aggregate body wt. during period III (g.)	Change in aggregate body wt. during period III (g.)
1	2	1	Normal	24	15	20	337	258·0	+24
1*a*	2	1	Normal	15	6	19	364	294	− 2
2	2	1	Pteroylglutamic acid-deficient	24	15	20	309	242·5	+ 5
3	2	—	Vitamin B_{12}-deficient	24	15	20	235	184·5	+21
3*a*	—	1	Vitamin B_{12}-deficient	30	15	20	130	102·5	+11
4	1*	1	(Pteroylglutamic acid + vitamin B_{12})-deficient	28	13	20	222	200·0	−10
5	1	—	Normal	30	7	14	145	148·5	+25
5*a*	1	—	Normal	30	7	14	159	151	+20
6	1	—	Pteroylglutamic acid-deficient	30	7	14	99	127	− 2
6*a*	1	—	Pteroylglutamic acid-deficient	30	7	14	74	124	−10
7	1	—	Pteroylglutamic acid-deficient during periods I and II, normal during period III	30	7	14	148	157·5	+35
8	1	1	Normal	17	9	14	301	278	+44
9	—	1	Normal	17	9	14	136	130·5	+47
10	2	1	Normal	15	10	13	452	421·5	+53
11	1	1	Normal	0	8	21	413	198	+120
12	1	1	Vitamin B_{12}-deficient	0	8	21	258	146·5	+35

* One animal died in this experiment.

RESULTS

Details of the food consumption and changes in body weight of the animals used in Expts. nos. 1–12 are given in Table 1. None of the animals in Expts. nos. 1–4 gained weight at a normal rate. In subsequent experiments, in which new batches of vitamins and amino acids were used for the diets, isotope ratios. These values range from 1·19 to 1·42 for normal animals, but are about double these figures (2·65 to 2·98) in pteroylglutamic acid deficiency. Similar differences were found in the incorporation of isotope from [^{15}N]glycine into alanine, aspartic acid and glutamic acid, although with these amino acids the effect of pteroylglutamic acid deficiency was less marked (Table 3).

Vitamin B_{12} deficiency. Neither the glycine/serine isotope ratio (Table 2) nor the incorporation of ^{15}N from labelled glycine into alanine, aspartic acid and glutamic acid (Table 3) was affected by lack of dietary vitamin B_{12}. In one experiment, in which 2% of [α-^{14}C]glycine was fed, the results with [^{15}N]glycine were confirmed (Table 4). The glycine/serine radioactivity ratio was 0·77 and 0·82 in normal and deficient animals respectively, and the incorporation of ^{14}C into alanine, aspartic acid and glutamic acid was similar in the presence and absence of dietary vitamin B_{12}.

Ascorbic acid deficiency. Normal and scorbutic guinea pigs showed similar incorporation of ^{14}C from [α-^{14}C]glycine into hippuric acid (Table 5). Moreover, the formation of glycine, isolated as hippuric acid, and formate from uniformly labelled L-[^{14}C]serine (Table 6) and the incorporation of

Table 2. *Conversion of [^{15}N]glycine into serine in normal, pteroylglutamic acid-deficient or vitamin B_{12}-deficient rats*

Details of the diets are given in the Experimental section. The ^{15}N content of the fed glycine was 15·76 atom % excess in Expts. nos. 1, 2, 3, 3*a* and 4, 15·08 in Expts. nos. 1*a* and 10, 16·29 in Expts. nos. 8 and 9, and 32·65 in Expts. nos. 5–7.

Expt. no.	Diet	^{15}N content of isolated amino acid (atom % excess)		Isotope ratio
		Glycine (*A*)	Serine (*B*)	*A*/*B*
1	Normal	0·550	0·432	1·27
1*a*	Normal	0·491	0·425	1·19
2	Pteroylglutamic acid-deficient	0·950	0·319	2·98
3+3*a*	Vitamin B_{12}-deficient	0·493	0·434	1·14
4	(Pteroylglutamic acid + vitamin B_{12})-deficient	0·673	0·253	2·66
5	Normal	1·096	0·863	1·27
5*a*	Normal	1·110	0·842	1·32
6	Pteroylglutamic acid-deficient	1·139	0·417	2·73
6*a*	Pteroylglutamic acid-deficient	1·114	0·432	2·65
7	Normal*	1·100	0·845	1·30
8	Normal	0·527	0·372	1·42
9	Normal	0·516	0·369	1·40
10	Normal	0·559	0·429	1·30

* This animal had been kept on deficient diets for 37 days (see Table 1).

Table 3. *Incorporation of ^{15}N from dietary [^{15}N]glycine into alanine, aspartic acid and glutamic acid in normal, pteroylglutamic acid-deficient and vitamin B_{12}-deficient rats*

Details as given in Table 2, and Experimental section. The isotope content of the isolated glycine is given in Table 2. — Signifies value not determined.

Expt. no.	Diet	^{15}N content of isolated amino acids (atom % excess)			Isotope ratio $\frac{\text{Isolated glycine}}{\text{Isolated amino acid}}$		
		Alanine	Aspartic acid	Glutamic acid	Alanine	Aspartic acid	Glutamic acid
1	Normal	—	0·040	0·054	—	13·7	10·2
1*a*	Normal	0·037	0·042	0·048	13·3	11·7	10·2
2	Pteroylglutamic acid-deficient	0·034	0·033	0·040	27·9	28·8	23·8
3+3*a*	Vitamin B_{12}-deficient	0·044	0·051	0·051	17·6	17·0	14·5
4	(Pteroylglutamic acid + vitamin B_{12})-deficient	0·028	0·029	0·034	24·0	23·2	19·8
5	Normal	0·068	0·084	0·070	16·1	13·0	15·6
5*a*	Normal	0·067	0·062	0·077	16·4	17·7	14·3
6	Pteroylglutamic acid-deficient	0·053	0·055	0·058	21·5	20·8	19·7
6*a*	Pteroylglutamic acid-deficient	0·048	0·045	0·054	23·9	25·4	21·2
7	Normal*	0·070	0·068	0·077	15·7	16·2	14·3
8	Normal	0·034	0·037	0·047	15·5	14·2	11·1
9	Normal	0·033	0·047	0·046	15·7	11·0	11·2
10	Normal	0·035	0·034	0·051	15·9	16·4	10·9

* This animal had been kept on deficient diets for 37 days (see Table 1).

[α-^{14}C]glycine into serine (Table 7) were not decreased significantly in ascorbic acid-deficient animals, showing that ascorbic acid had no detectable effect on glycine metabolism in these experiments.

Rate of glycine biosynthesis in normal and deficient animals

The average amount of glycine synthesized by the animals in Expts. nos. 1–14 (x, in mg./100 g. body wt./day) has been calculated from the equation (see Arnstein & Neuberger, 1953*b*) $x = 100a(m/n - 1)/w$, where a is the average amount of labelled glycine ingested (mg./day, calculated from Tables 1 and 7), w is the mean aggregate body weight (Tables 1 and 7) and m and n are the isotope contents of the fed and isolated glycine respectively (Tables 2 and 7).

Table 8 shows that the rate of glycine biosynthesis by the guinea pig is very similar to that by the rat, especially since the rats were fed on serine-free diets whilst no allowance has been made for the amount of serine in the guinea-pig diet. Ascorbic acid deficiency does not appear to have any significant effect on the rate of glycine synthesis by the guinea pig, although only a limited number of results are available. From a statistical analysis of all experiments done with animals from the same litter, including some of those reported earlier (Arnstein & Neuberger, 1953*b*), it is clear that vitamin B_{12} has no effect on glycine biosynthesis (variance ratio less than 1, Table 9). By contrast, a deficiency of pteroylglutamic acid results in a marked decrease in the amount of glycine synthesized by the rat, the variance ratio being significant at the 0·1 % level. The effect of pteroylglutamic acid deficiency on glycine biosynthesis is largely independent of the growth of the animals, as indicated by a comparison of the results of Expts. nos. 1–4 with those of nos. 5–7.

Table 4. *Conversion of* [α-^{14}C]*glycine into other amino acids in normal and vitamin* B_{12}*-deficient rats*

[α-^{14}C]Glycine (2%) was fed as a component of diet AAD 2 for 21 days (Arnstein & Neuberger, 1953*a*). The specific radioactivities of the isolated amino acids have been calculated on the basis of the radioactivity of the fed glycine = 1 mc/mole. For further details of diet and animals see Experimental section and Table 1, Expts. 11 and 12.

	Radioactivity (μc/mole)	
Amino acid isolated	Normal diet	Vitamin B_{12}-deficient diet
Alanine	18·5	17·4
Aspartic acid	23·0	23·9
Glutamic acid	28·0	29·7
Glycine	539	492
Serine	703	604

Table 5. *Hippuric acid synthesis by normal and scorbutic guinea pigs*

In Expt. no. 15, the animals were kept on the experimental diets for 15 days. Each animal was then injected intraperitoneally with an aqueous solution (2 ml./100 g. body wt.) containing DL-serine (126·0 mg.), sodium benzoate (87·0 mg.) and [α-^{14}C]glycine (0·4 μc; 1·36 mg.)/2 ml. In Expt. no. 16 the guinea pigs were kept on the experimental diets for 20 days and each animal was injected intraperitoneally with a solution (2 ml./100 g. body wt.) containing DL-serine (65·6 mg.), sodium benzoate (44·9 mg.) and [α-^{14}C]glycine (0·48 μc; 0·58 mg.)/2 ml. Ascorbic acid was fed at the level of 5 mg./animal/day. Urine was collected for 24 hr. — Signifies value not determined.

Expt. no.	Diet	Animal no.	Body wt. (g.) Initial	Body wt. (g.) Final	Specific radioactivity of hippuric acid (μc/g.)
15	41	1	209	228	0·856
		2	183	212	0·823
		3	185	234	0·693
		4	185	223	0·872
		5	191	207	1·047
15	41 + ascorbic acid	6	208	276	0·702
		7	202	291	0·892
		8	201	273	0·871
15	18 + cabbage	9	191	285	0·710
		10	185	296	0·880
16	41	11	203	232	1·40
		12	212	210	1·63
16	41 + ascorbic acid	13	209	192	1·78
16	18 + cabbage	14	—	240	1·44
		15	—	227	1·39
		16	—	237	1·55

Table 6. *Conversion of serine into glycine and formate by normal and scorbutic guinea pigs*

In Expt. no. 17, six guinea pigs were kept on diet S for 26 days with or without ascorbic acid (5 mg./animal/day). Each animal was then injected intraperitoneally with sodium benzoate (86·4 mg./100 g. body wt.) and on the following day with an aqueous solution (2 ml./100 g. body wt.) containing L-[^{14}C]serine (16 mg.; 0·35 μc) and sodium benzoate (43·2 mg.)/2 ml. In Expt. no. 18, the deficient animals were kept 18 days on diet 41, the normal animals on diet 18 + cabbage. Each animal was given an intraperitoneal injection of a solution (2·5 ml./100 g. body wt.) containing sodium formate (136 mg.), sodium benzoate (92·4 mg.), DL-serine (32 mg.) and L-[^{14}C]serine (0·35 μc, specific radioactivity approx. 25 μc/mg.)/2·5 ml. Urine was collected for 6 hr. The radioactivity of the formate was determined after conversion into $BaCO_3$ (see Experimental section). — Signifies value not determined.

Expt. no.	Diet	Animal no.	Body wt. (g.) Initial	Body wt. (g.) Final	Specific radioactivity (μc/g.) Hippuric acid	Specific radioactivity (μc/g.) Formate (as $BaCO_3$)
17	S	17	291	269	0·708	—
		18	324	295	0·937	—
		19	300	236	0·639	—
17	S + ascorbic acid	20	285	320	0·787	—
		21	287	308	0·773	—
		22	300	263	0·935	—
18	41	23	402	305	0·225	0·0154
		24	254	204	0·357	0·0128
18	18 + cabbage	25	—	304	0·337	0·0132
		26	—	296	0·435	0·0154

Table 7. *Conversion of glycine into serine in normal and scorbutic guinea pigs*

Diet 41 contained 1·33% [α-^{14}C]glycine (1·04 μc/g.) and was fed for 23 days with or without ascorbic acid (5 mg./animal/day). Results have been calculated for a specific radioactivity of dietary glycine = 1 mc/mole.

Expt. no.	Animal no.	Diet	Body wt. (g.) Initial	Body wt. (g.) Final	Average food consumption (g./day)	Radioactivity of isolated amino acids (μc/mole) Glycine (*A*)	Radioactivity of isolated amino acids (μc/mole) Serine (*B*)	Isotope ratio: *A*/*B*
13	1	41	348	235	9·2	132	155	0·85
	2	41	373	256	8·8	110	114	0·96
14	3	41 + ascorbic acid	374	346	11·0	121	115	1·06
	4	41 + ascorbic acid	348	282	10·2	124	143	0·87

DISCUSSION

It is known that a reduced form of pteroylglutamic acid, probably the tetrahydro derivative (Blakley, 1954; Kisliuk & Sakami, 1954), is required for the metabolic interconversion of glycine and serine. Ascorbic acid increases the *in vitro* synthesis of the closely related N^5-formyltetrahydropteroylglutamic acid (citrovorum factor) (Nichol, 1953), and treatment of scorbutic patients with ascorbic acid restored normal excretion of citrovorum factor after oral administration of pteroylglutamic acid (Gabuzda, Phillips, Schilling & Davidson, 1952). It was considered possible, therefore, that ascorbic acid deficiency may decrease the biosynthesis of the metabolically active form of pteroylglutamic acid and thus result in a derangement of glycine metabolism. The present experiments indicate, however, that scorbutic guinea pigs retain the ability to synthesize normal amounts of glycine and that the serine–glycine interconversion is likewise not reduced. The marked decrease in the free glycine content of muscle of scorbutic guinea pigs which has been observed under conditions essentially similar to those used in the present experiments (Christensen & Lynch, 1948) is thus probably not due to a decrease in glycine biosynthesis. Moreover, the concentration of glycine in liver is not affected by ascorbic acid deficiency (Christensen & Lynch, 1948), and the effect of scurvy on muscle glycine may thus be caused by a specific alteration in the permeability of muscle cells to glycine rather than by a disturbance of glycine metabolism.

Although the levels of ascorbic acid in the liver and other internal organs of scorbutic guinea pigs are markedly lower than those of normal animals (Weingärtner, 1942), the present results are not necessarily incompatible with a function of ascorbic acid in the formation of the metabolically active pteroylglutamic acid derivative, since very low levels of ascorbic acid may be sufficient for this reaction and hence for normal glycine metabolism.

The utilization of $^{15}NH_3$ for hippuric acid synthesis is much more efficient in the guinea pig than in the rat (Shemin, 1946), but the rate of glycine biosynthesis is surprisingly similar in both species (Table 8). No allowance has been made for the presence of serine in the guinea-pig diets, but in the absence of dietary serine the figures for glycine biosynthesis by the guinea pig would have been even lower. It is noteworthy that, contrary to an earlier suggestion (Arnstein, 1954), differences in the utilization of ammonia for hippuric acid synthesis are apparently not related to the rate of glycine biosynthesis.

In the experiments with vitamin B_{12} no special attempt was made to deplete the animal's stores, and it is probable therefore that the deficiency was not severe. Nevertheless, the administration of oral vitamin B_{12} resulted in a significant increase in the growth rate when a labile-methyl-deficient diet was used (Expts. nos. 8 and 9, Table 1, and earlier experiments by Arnstein & Neuberger, 1953*a*). No effect on the gain in body weight was observed with diets containing methionine (Expts. nos. 3 and 3*a*, Table 1). It is clear, however, that both the glycine–serine reaction and the rate of glycine biosynthesis was unaffected by vitamin B_{12}. These results, which are in agreement with our earlier work (Arnstein & Neuberger, 1953*a*), do not support the suggestion that vitamin B_{12} deficiency interferes with the conversion of glycine into serine. The reduced utilization of [α-^{14}C]glycine for the biosynthesis of the cysteine and glutamic acid moieties of glutathione in vitamin B_{12} deficiency (Anderson & Stekol, 1953) is thus probably not due to a decreased formation of serine from glycine.

It has been known for some time that pteroylglutamic acid is required for the interconversion of serine and glycine (Elwyn & Sprinson, 1950; Totter, Kelley, Day & Edwards, 1950). The present experiments show that pteroylglutamic acid deficiency considerably decreases the rate of glycine biosynthesis as well as the glycine–serine reaction. This effect is not due to the poorer growth of the deficient rats, as shown by comparison of Expts. nos. 1 and 1*a* with 2, and 5 and 5*a* with 6 and 6*a*, in all of which glycine biosynthesis was less in the deficient animals. It seems that this marked decrease in glycine biosynthesis is an early metabolic manifestation of pteroylglutamic acid deficiency, since it occurred as soon as changes in

Table 8. *Glycine biosynthesis by the rat and guinea pig on various diets*

For diets and other experimental details see text.

Expt. no.	Diet	Glycine biosynthesis (mg./day/100 g. body wt.)
	Rat	
1	Normal	181
1*a*	Normal	194
2	Pteroylglutamic acid-deficient	99
3+3*a*	Vitamin B_{12}-deficient	197
4	(Pteroylglutamic acid and vitamin B_{12})-deficient	124
5	Normal	201
5*a*	Normal	224
6	Pteroylglutamic acid-deficient	155
6*a*	Pteroylglutamic acid-deficient	117
7	Normal*	199
8	Normal	231
9	Normal	228
10	Normal	214
11	Normal	178
12	Vitamin B_{12}-deficient	173
	Guinea pig	
13	Normal	277
13	Normal	299
14	Ascorbic acid-deficient	296
14	Ascorbic acid-deficient	304

* The animal in this experiment was kept on pteroylglutamic acid-deficient diets during the preliminary feeding periods (see Tables 1 and 2).

Table 9. *Effect of deficiencies of pteroylglutamic acid and vitamin B_{12} on glycine biosynthesis by the rat*

Only experiments in which pairs of animals from the same litter were used have been included. The first four pairs of values for vitamin B_{12}-deficient and corresponding normal rats refer to previously published results (Table 4, Expts. nos. 1, 3, 4, 5, 7, 8, 10 and 11*b* respectively, Arnstein & Neuberger, 1953*b*). The numbers of the present series of experiments (see Table 1 and Experimental section for details) are given in parentheses.

Glycine biosynthesis (m-moles/day/100 g. body wt.)			
Normal diet	Vitamin B_{12}-deficient diet	Normal diet	Pteroylglutamic acid-deficient diet
2·84	2·52	2·41 (1)	1·32 (2)
2·79	2·79	2·62 (3+3*a*)*	1·65 (4)*
2·63	2·80	2·68 (5)	2·06 (6)
3·09	2·41	2·98 (5*a*)	1·56 (6*a*)
2·37 (11)	2·30 (12)	—	—
2·41 (1)	2·63 (3+3*a*)	—	—
Mean ± S.E.M. **2·69 ± 0·11**	**2·58 ± 0·08**	**2·67 ± 0·12**	**1·65 ± 0·15**

* Diet was also vitamin B_{12}-deficient.

body weight and leucocyte counts became apparent (Expts. nos. 5 and 6 and Fig. 1). The amount of glycine synthesized by the growing rat under normal conditions apparently exceeds its requirements by only a small margin (Arnstein & Neuberger, 1953*b*). In pteroylglutamic acid deficiency glycine biosynthesis takes place at only about 50–60% of the normal rate and it seems possible that under these conditions glycine may become virtually an 'essential' amino acid.

It has been suggested that serine is the most important source of endogenous glycine and also quantitatively the most important precursor of the one-carbon compounds related to formate (Arnstein, 1954). The observation that pteroylglutamic acid deficiency reduces both the serine–glycine interconversion and glycine biosynthesis to about the same extent lends support to this view and may also explain the delay in the incidence of pteroylglutamic acid deficiency in rats fed on diets containing choline or betaine (Stekol, Weiss, Smith & Weiss, 1953). Both compounds are metabolized to glycine and one-carbon compounds by reactions which, as far as is known, do not require pteroylglutamic acid and may thus be utilized by the deficient animal in place of serine.

SUMMARY

1. Diets containing known amounts of isotopically labelled glycine were fed to rats or guinea pigs for periods of 13–21 days and the isotope content of the glycine isolated from the protein of the mixed viscera at the end of the experiment was determined. The food consumption and changes in body weight were also measured.

2. The amount of glycine synthesized by the two species under normal conditions and in the absence of certain vitamins was calculated from the dilution of the fed glycine.

3. Normal glycine biosynthesis by the rat and the guinea pig was 2·7 and 4 m-moles/100 g. body weight/day respectively.

4. Vitamin B_{12}-deficient rats or ascorbic acid-deficient guinea pigs synthesized normal amounts of glycine. Ascorbic acid also had no effect on the synthesis of hippuric acid and formate from serine.

5. Pteroylglutamic acid deficiency resulted in a marked decrease in glycine biosynthesis by the rat and in a reduced conversion of glycine into serine, alanine, aspartic acid and glutamic acid.

6. These results are discussed with reference to the importance of serine and pteroylglutamic acid for glycine biosynthesis.

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A Comparison of Alanine and Glucose as Precursors of Serine and Glycine

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The importance of glycine in animal and plant metabolism as a precursor of many nitrogenous compounds, such as creatine, porphyrins, chlorophyll, uric acid and nucleic acid purines is now well established (for a recent review, see Arnstein, 1954). Relatively little is known, however, about the metabolic origin of this amino acid from non-nitrogenous precursors. That glycine can be synthesized by mammals has been recognized for almost half a century (Magnus-Levy, 1907; Wiechowski, 1906), but conclusive proof that it is a nutritionally dispensable amino acid has been obtained only recently in feeding experiments with synthetic diets (Rose, Burr & Sallach, 1952).

The metabolism of glycine is closely linked to that of serine, since both amino acids are readily converted into one another both *in vivo* (Shemin, 1946; Sakami, 1948) and *in vitro* (Blakley, 1954; Kisliuk & Sakami, 1954). The biosynthesis of either serine or glycine from three-carbon or two-carbon compounds in sufficient amounts would therefore be adequate for the animal's requirements, and both mechanisms have in fact been proposed for the biosynthesis of glycine. Thus, the conversion of [2-^{14}C] and [1-^{13}C]pyruvate into glycine *in vivo* has been reported (Anker, 1948), and it was suggested that serine was formed as an intermediate. More recently it has been shown that glycollic acid (Weinhouse & Friedmann, 1951; Chao, Delwiche & Greenberg, 1953) and glyoxylic acid (Weinhouse & Friedmann, 1951; Weissbach & Sprinson, 1953) can be used for the biosynthesis of glycine, presumably by reductive amination of glyoxylic acid. It is not clear, however, whether these C_2 acids are formed in sufficient amounts to account quantitatively for glycine biosynthesis; the available evidence concerning the relative importance of C_3 and C_2 precursors has so far been inconclusive, although it has been shown that, on a diet free of both serine and glycine, the biosynthesis of serine by the rat significantly exceeds that of glycine (Arnstein & Neuberger, 1953*b*).

The recent observation that the biosynthesis of glycine from non-nitrogenous precursors is markedly reduced in pteroylglutamic acid deficiency (Arnstein & Stanković, 1956) suggested to us that it should be possible to differentiate between these alternative mechanisms by comparing the effect of pteroylglutamic acid deficiency on the biosynthesis of glycine and serine from a non-nitrogenous precursor, such as glucose, since this vitamin deficiency reduces the metabolic interconversion of the two amino acids (Elwyn & Sprinson, 1950*b*; Totter, Kelley, Day & Edwards, 1950). The results of such experiments, which are now reported, show that the biosynthesis of serine from glucose or alanine is independent of the glycine–serine reaction, whilst an important part of glycine must be synthesized from serine. Neither alanine nor pyruvate, which arises from it by transamination, were efficient serine precursors when compared with glucose, and it is suggested that serine is formed from a C_3 precursor which is closely related to an intermediate of glycolysis, such as a triose or glyceric acid. Some of these results have already been reported in a preliminary communication (Arnstein, 1955).

EXPERIMENTAL

Animals and diets

Albino rats of the National Institute for Medical Research (Institute) stock were placed on a 12% casein diet containing 1% of succinylsulphathiazole (Arnstein & Stanković, 1956) soon after weaning for periods varying from 15 to 30 days. The synthetic amino acid diet AAD3 (Arnstein & Stanković, 1956) was then given for 6–15 days, and finally the same diet, but containing [^{15}N]glycine and either L-[^{14}C]alanine (0·59%) or D-[^{14}C]glucose (34%) was fed, usually for either 14 or 20 days, as indicated in Tables 1 and 2. All diets were supplemented with the usual vitamins in the amounts given previously (Arnstein & Stanković, 1956), except that vitamin B_{12} or pteroylglutamic acid was omitted in the experiments with deficient animals. Full details of the food consumption and changes in body weight of these animals have already been given (Expts. nos. 1–10, Table 1, Arnstein & Stanković, 1956) and the earlier experiment numbers are retained throughout this paper.

Labelled compounds

Uniformly labelled L-[^{14}C]*alanine and* D-[^{14}C]*glucose.* These compounds were purchased from the Radiochemical Centre, Amersham.

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D-[1-^{14}C]*Glucose*. The glucose used in Expt. no. 9 was synthesized following the method of Sowden (1949). The initial conversion of [^{14}C]methyl iodide into [^{14}C]nitromethane was carried out in the presence of unlabelled nitromethane (cf. Arnstein & Bentley, 1951). In Expt. no. 5*a* D-[1-^{14}C]glucose obtained from the National Bureau of Standards, Washington, U.S.A., was used.

L-[β-^{14}C]*Alanine*. L-Alanine was prepared by condensing methyl iodide with the sodium derivative of ethyl benzamidomalonate (Redemann & Dunn, 1939), hydrolysis of the ester and decarboxylation to benzoylalanine, which was resolved as the strychnine salt (Pacsu & Mullen, 1940).

(*a*) *Trial experiments*. To the sodium derivative of ethyl benzamidomalonate in dry ethanol (7 ml.), prepared from sodium (0·0605 g./2 ml. of ethanol) and ethyl benzamidomalonate (0·734 g./6 ml. of ethanol), methyl iodide (0·355 g.) was added and the solution was heated in a sealed tube at 90–100° for 3 hr. The ethanol was evaporated and the dry residue was dissolved in $CHCl_3$ (30 ml.) and NaI removed by filtration. The filtrate was evaporated to dryness, giving crude ethyl benzamidomethylmalonate (0·588 g., 80%), which was dissolved in ethanol (8 ml.) and N-NaOH (4 ml.) was added. After 3 hr. at room temperature the ethanol was evaporated and N-HCl (4 ml.) was added. The solution was evaporated to dryness and the residue was decarboxylated by heating for 1 hr. at 120–125° in a stream of N_2. The product was dissolved in $CHCl_3$ (5 ml.) and purified by partition chromatography on Celite (50 g.), using 0·5 N-H_2SO_4 (25 ml.) as the stationary phase and $CHCl_3$ as the mobile phase. The effluent (30 ml. fractions) was collected in a titration vessel through which CO_2-free N_2 was passed and was titrated with 0·02 N-KOH in methanol, using cresol red as indicator. Benzoylalanine was eluted in fractions nos. 5–13 (peak in nos. 6–8; total titre: 60·0 ml. 0·02 N-KOH corresponding to a 49% yield based on methyl iodide. In two other experiments with half these quantities the yields of benzoyl-DL-alanine at this stage were 38 and 39%). The fractions were evaporated to dryness, the residue was dissolved in water (25 ml.) and the pH adjusted to 7. The calculated amount of strychnine based on the titration (200 mg.) was added and dissolved by boiling. After treatment with a little charcoal the solution was filtered and kept at 0° for 3 days. Yield of benzoyl-L-alanine strychnine salt: 250 mg. (79%), m.p. 128–129°.

(*b*) *Radioactive preparation*. The L-[β-^{14}C]alanine was synthesized from [^{14}C]methyl iodide (approx. 1·75 mc; 0·75 g.) by an essentially similar procedure. The condensation was, however, much less satisfactory than in the trial experiment, as judged by a comparison of the chemical and radiochemical yields and by the presence of glycine in the final product. It seems possible that the [^{14}C]methyl iodide may have deteriorated owing to storage for over a year. The following modifications of the trial method were adopted. The crude N-benzoylalanine appeared to be insoluble in $CHCl_3$. It was dissolved in *n*-butanol (5 ml.), $CHCl_3$ (10 ml.) was added and the solution chromatographed on Celite (100 g.), using 2% (v/v) butanol–$CHCl_3$ as the mobile phase. Fractions 4–19 required 115 ml. of 0·02 N-KOH, equivalent to 444 mg. of benzoylalanine (43·5%). These fractions were evaporated to dryness, carrier benzoylalanine (444 mg.) was added and the strychnine salt of benzoyl-L-alanine prepared as above. Yield 877·6 mg. (72%), m.p. 127–129°. The strychnine salt was dissolved in the minimum amount of hot water, N-KOH (1·7 ml.) was added, the solution cooled and strychnine removed by filtration. N-HCl (1·7 ml.) was added to the filtrate, which was then concentrated to approx. 2 ml., when benzoyl-L-[β-^{14}C]alanine crystallized. After recrystallization from ethyl acetate–light petroleum (b.p. 60–80°) the product (270 mg., 84%) had m.p. 143–147°; $[\alpha]_D^{20}$ of K salt 29·9 ± 0·7 in water (*c*, 2·065). Under the same conditions non-radioactive benzoyl-L-alanine had m.p. 147–148°; $[\alpha]_D^{20}$ + 31·1°. The specific radioactivity of this material was 0·195 μc/mg. (theoretical specific radioactivity, calculated from chemical yields and radioactivity of starting material = 0·84 μc/mg.).

Benzoyl-L-[β-^{14}C]alanine (250 mg.) was refluxed with 3 N-HCl (10 ml.) for 2 hr. The solution was concentrated to dryness, the residue dried *in vacuo* (P_2O_5) and benzoic acid was removed by extraction with light petroleum (b.p. 60–80°). The hydrochloride was dissolved in ethanol (0·9 ml.) and L-[β-^{14}C]alanine precipitated with pyridine (0·15 ml.). Yield: 69 mg. (60%); $[\alpha]_D^{20}$ 10·2 ± 1·4 in N-HCl (*c*, 0·52); specific radioactivity, 0·345 μc/mg. A second crop of L-[β-^{14}C]alanine was obtained from the mother liquors by adsorption of the amino acid on Zeo-Karb 215 resin (Permutit Co. Ltd.) and elution with 0·02 M-ammonia (27 mg. 0·27 μc/mg.). The purity of the L-[β-^{14}C]alanine was checked by chromatographing 0·25 mg. on Whatman no. 3 paper, using amyl alcohol–pyridine–water (7:7:6, v/v). Both crops contained traces of glycine, which was, however, inactive.

For use in the diets the fractions (39·5 mg. of the main crop, 26·0 mg. of the second crop) were combined and diluted with carrier L-alanine (12·5 g.). The specific radioactivity of the diluted L-[β-^{14}C]alanine was 1·84 μc/g.

Isolation of amino acids

At the end of the experimental feeding period the animals were killed under anaesthesia and the internal organs (heart, lungs, liver, spleen, reproductive organs and kidneys) were minced, extracted six times with boiling ethanol, three times with trichloroacetic acid (6%, w/v) and again with ethanol and ether, and dried at about 80°. The crude protein was hydrolysed with 6 N-HCl (10 ml./g.) at 100° for about 20 hr. in a sealed tube. The required amino acids were isolated by chromatography on Zeo-Karb 225 resin (Permutit Co., Ltd.) from this hydrolysate as described by Arnstein & Neuberger (1953*b*). In some experiments, serine (Expts. nos. 1, 2 and 4) and glycine (Expt. no. 1) were isolated as the *N*-benzoyl derivatives by method A of Arnstein & Neuberger (1953*b*). In the other experiments paper chromatography was used to purify amino acids which had been incompletely separated on the ion-exchange column (Arnstein & Stanković, 1956). The purity of the isolated amino acids was checked by paper chromatography and constant radioactivity after recrystallization.

Degradation of serine, glycine and glutamic acid. Serine was degraded with periodate by the procedure of Sakami (1950), as modified by Arnstein & Neuberger (1953*b*). Glycine was decarboxylated with ninhydrin (Arnstein, 1951) to give formaldehyde and CO_2, which were counted as the dimedon derivative and $BaCO_3$ respectively. Glutamic acid was decarboxylated with Chloramine T (Mosbach, Phares & Carson, 1951; Dakin, 1917). CO_2 arising from $C_{(1)}$ was collected as $BaCO_3$ and γ-oxobutyric acid from carbon atoms 2–5 was counted as the 2:4-dinitrophenylhydrazone derivative, m.p. 198–199°.

Radioactivity measurements. Samples of 'infinite thickness' were plated on 1 cm.^{2} polythene disks (Popják, 1950) and counted with a bell-shaped helium-filled Geiger–Müller counter. The combined errors due to variations in sample preparation and statistical errors were usually about $\pm 5\%$, but the very low radioactivities of the glycine and serine samples in Expts. nos. 1–4 and no. 10 resulted in errors of about $\pm 10\%$.

RESULTS

In order to compare the results of different experiments the radioactivities of the isolated amino acids have been calculated for a specific radioactivity (= 1 mc/mole) of the labelled alanine or glucose fed in the diet.

The incorporation of ^{14}C from dietary [^{14}C]-alanine into serine, glycine, alanine, aspartic acid and glutamic acid isolated from the protein of the mixed viscera is shown in Table 1. The radioactivities of alanine, aspartic acid and glutamic acid are of the same order of magnitude, whilst those of serine and glycine are much lower. When uniformly labelled alanine was fed (Expt. no. 10) this difference between the radioactivities of alanine, aspartic acid and glutamic acid, on the one hand, and serine and glycine, on the other, was even more marked, but it should be noted that the labelled compound was fed for a considerably shorter period in this experiment (13 days compared with 19 or 20 days in Expts. nos. 1–4).

Although the very low radioactivities of the isolated serine and glycine do not permit a very accurate comparison, the alanine/glycine and alanine/serine radioactivity ratios in comparable experiments (Table 1, Expts. nos. 1*a*–4) suggest that vitamin B_{12} has little or no effect on the conversion of alanine into either glycine or serine. Pteroylglutamic acid deficiency, however, reduced its conversion into glycine, whilst that into serine appears to be substantially unchanged. The incorporation of alanine into glutamic acid and aspartic acid is also similar in all experiments.

Table 2 shows the conversion of dietary glucose, labelled either in $C_{(1)}$ or in all C atoms ([U-^{14}C]-glucose), into serine, glycine, alanine, aspartic acid and glutamic acid of the visceral proteins under conditions which are essentially comparable to those of the experiment with uniformly labelled alanine (Expt. no. 10, Table 1). The radioactivities of the isolated alanine were remarkably similar in all experiments, whilst both aspartic acid and glutamic acid contained more radioactivity when [1-^{14}C]glucose was fed. The conversion of glucose into serine was also similar in all experiments and was not affected by pteroylglutamic acid deficiency. Glycine, however, contained much less radioactivity in the absence of pteroylglutamic acid or in the [1-^{14}C]glucose experiments. It is particularly noteworthy that the difference in radioactivity of the isolated alanine and serine was much less than in the experiments with labelled alanine. Thus, the average radioactivity of serine in Expts. nos. 6–9 was 76% of that of alanine, whilst in the comparable experiment with uniformly labelled alanine serine had only 15% of the radioactivity of the isolated alanine.

Table 1. *Conversion of* L-[β-^{14}C]*alanine and uniformly labelled* [^{14}C]*alanine into serine, glycine and some other amino acids*

The experimental diet contained 0·59% of labelled alanine and was fed for 19 days (Expt. no. 1*a*), 20 days (Expts. nos. 1–4) or 13 days (Expt. no. 10). The specific radioactivities of the dietary L-[β-^{14}C]alanine and the uniformly labelled [^{14}C]alanine were 164 and 991 μc/mole respectively, but all results have been calculated for a specific radioactivity of the fed labelled compounds = 1 mc/mole in order to facilitate comparison of different experiments. For further experimental details see text and Arnstein & Stanković (1956).

Expt. no.	Diet	Labelled compound fed	Radioactivity of isolated amino acids (μc/mole)					Radioactivity ratios	
			Serine	Glycine	Alanine	Aspartic acid	Glutamic acid	Alanine / serine	Alanine / glycine
1	Normal	L-[β-^{14}C]-alanine	4·1	2·1	—*	8·4	14·1	—	—
1*a*	Normal		2·8	1·65	10·4	10·7	14·9	3·7	6·3
2	Pteroylglutamic acid-deficient		3·6	1·4	13·5	10·2	16·1	3·7	9·6
3+3*a*	Vitamin B_{12}-deficient		3·1	1·5	9·5	9·6	14·1	3·1	6·3
4	(Pteroylglutamic acid + vitamin B_{12})-deficient		3·6	0·94	9·8	10·6	14·2	2·7	10·5
10	Normal	Uniformly labelled L-[^{14}C]-alanine	1·5	0·97	10·0	7·9	10·8	6·7	10·3

* Sample lost by accident.

The distribution of ^{14}C in the isolated serine, glycine and glutamic acid is given in Table 3. Apart from the radioactivity of the serine carboxyl C in Expt. no. 6 and of the β-C in Expt. 10, which are somewhat low, uniformly labelled glucose or alanine gives rise to a uniform distribution of ^{14}C in serine, glycine and, as far as can be seen from the partial degradation, in glutamic acid. In both experiments with [1-^{14}C]glucose, on the other hand, significantly more ^{14}C was incorporated into the β-position of serine than into the other carbon atoms, and the α-C of glycine was also somewhat more radioactive than the carboxyl C atom. The absolute radioactivity of the carboxyl carbon atom of the glutamic acid derived from [1-^{14}C]glucose (Expt. no. 9) was similar to that in the experiments with uniformly labelled glucose, although in the latter experiments rather more of the total radioactivity was located in this position.

DISCUSSION

Effect of pteroylglutamic acid deficiency on glycine and serine biosynthesis

In normal animals the glycine–serine reaction (Shemin, 1946; Sakami, 1948) leads to the rapid metabolic interconversion of these two amino acids, and it is therefore difficult, if not impossible, to assess whether their biosynthesis from non-nitrogenous precursors takes place primarily by formation of serine from a C_3 precursor (Fig. 1, reaction 1), followed by conversion of serine into glycine (reaction 2) or, alternatively, by the synthesis of glycine from a C_2 precursor (reaction 3) and the subsequent addition of a one-carbon compound to give serine (reaction 2). Both reaction sequences may, of course, occur simultaneously, and it is of interest that glycollic acid (Weinhouse & Friedmann, 1951; Chao *et al.* 1953) and glyoxylic

Table 2. *Conversion of labelled glucose into serine, glycine, alanine, aspartic acid and glutamic acid*

The animals in Expts. nos. 5–7 were litter mates. For details of food consumption and changes in body weight see Arnstein & Stanković (1956). The experimental diets were fed for 14 days in all experiments. The diet in Expts. nos. 5–7 contained 0·59% of non-isotopic L-alanine, which was omitted in Expts. nos. 8 and 9. Results calculated for specific radioactivity of fed glucose = 1 mc/mole. Uniformly labelled D-[^{14}C]glucose is indicated by [U-^{14}C]glucose.

Expt. no.	Diet	Labelled compound fed	Radioactivity of isolated amino acid (μc/mole) Serine	Glycine	Alanine	Aspartic acid	Glutamic acid
6	Pteroylglutamic acid-deficient	[U-^{14}C]Glucose	101	33·6	128	104	140
6*a*	Pteroylglutamic acid-deficient	[U-^{14}C]Glucose	99·4	35·8	123	111	155
5	Normal	[U-^{14}C]Glucose	91·7	55·3	134	127	172
7	Normal*	[U-^{14}C]Glucose	100	57·2	127	129	168
8	Normal	[U-^{14}C]Glucose	119	69·8	131	135	188
5*a*	Normal	[1-^{14}C]Glucose	81·2	29·1	139	178	271
9	Normal	[1-^{14}C]Glucose	94·9	27·0	126	173	273

* The animal in this experiment was fed on pteroylglutamic acid-deficient diets (as in Expts. nos. 6 and 6*a*) for 37 days before the beginning of the experimental period.

Table 3. *Degradation of serine, glycine and glutamic acid isolated from animals fed on labelled glucose or alanine*

For experimental details see text. — Signifies value not determined. [U-^{14}C]Glucose and [U-^{14}C]alanine indicate uniformly labelled D-[^{14}C]glucose and L-[^{14}C]alanine respectively.

Expt. no.	Diet	Labelled compound fed	Distribution of ^{14}C in isolated amino acids (% of total) Serine β-C	Serine α-C	Serine Carboxyl C	Glycine α-C	Glycine Carboxyl C	Glutamic acid C_{2-5}	Glutamic acid α-Carboxyl C
5	Normal	[U-^{14}C]Glucose	—	—	—	40·2	42·7	—	—
5*a*	Normal	[1-^{14}C]Glucose	55·6	25·2	16·1	60·2	44·0	—	—
6	Pteroylglutamic acid-deficient	[U-^{14}C]Glucose	37·2	31·8	21·5	49·5	52·1	72·3	20·0
6*a*	Pteroylglutamic acid-deficient	[U-^{14}C]Glucose	—	31·8	32·4	—	—	75·3	19·7
7	Normal*	[U-^{14}C]Glucose	37·1	32·1	34·9	—	—	72·6	20·8
8	Normal	[U-^{14}C]Glucose	32·1	36·5	31·9	45·5	47·0	76·8	18·1
9	Normal	[1-^{14}C]Glucose	65·2	10·7	15·4	55·9	40·9	82·3	13·1
10	Normal	[U-^{14}C]Alanine	24·8	39·9	42·6	—	—	—	—

* See footnote, Table 2.

acid (Weinhouse & Friedmann, 1951; Weissbach & Sprinson, 1953) are available as C_2 precursors of glycine, whilst pyruvate can apparently serve as a C_3 precursor of serine (Anker, 1948). No information is available, however, to indicate which of these reactions predominates.

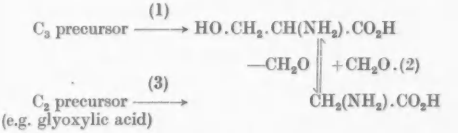

Fig. 1. Possible alternative metabolic pathways leading to the biosynthesis of glycine and serine.

There is abundant evidence that pteroylglutamic acid is required for the interconversion of serine and glycine both *in vivo* (Elwyn & Sprinson, 1950*b*; Totter *et al.* 1950) and *in vitro* (Blakley, 1954; Kisliuk & Sakami, 1954), as well as for glycine biosynthesis (Arnstein & Stanković, 1956), and these observations have now been applied to a study of the metabolic origin of glycine and serine. Since pteroylglutamic acid deficiency had no effect on the biosynthesis of serine from either glucose or alanine it is concluded that the glycine–serine reaction is not essential for serine biosynthesis from non-nitrogenous precursors. On the other hand, the conversion of both alanine and glucose into glycine in pteroylglutamic acid-deficient rats was only about 60 % of that in normal animals. It is particularly noteworthy that both the conversion of dietary [^{15}N]glycine into serine and the dilution of the fed labelled glycine by endogenously synthesized glycine were also reduced to a similar extent (Arnstein & Stanković, 1956). These results suggest that glycine is synthesized largely by the glycine–serine reaction and that serine is an important intermediate in the biosynthesis of glycine from non-nitrogenous precursors.

The β-carbon atom of serine appears to be a major precursor of 'active' formaldehyde, as shown by its importance as a source of methyl groups (Arnstein & Neuberger, 1953*a*). This reaction is also decreased by pteroylglutamic acid deficiency (Stekol, Weiss & Weiss, 1952), and it therefore seems reasonable to suppose that the biosynthesis of glycine and 'active' formaldehyde are linked by the glycine–serine reaction.

It is noteworthy that pteroylglutamic acid deficiency had no effect on the metabolism of glucose to pyruvate in the present experiments.

Utilization of glucose and alanine for the formation of serine and glycine

A comparison of the experiments with uniformly labelled [^{14}C]glucose and [^{14}C]alanine shows that glucose is utilized almost equally efficiently for the biosynthesis of serine, glycine and alanine, whilst alanine, which was used as a metabolic source of pyruvate, was a relatively poor precursor of serine and glycine.

This is not due to a preferential utilization of dietary alanine for other reactions, e.g. protein synthesis, since in all experiments there was extensive metabolism of this amino acid to glutamic acid and aspartic acid, presumably by transamination to pyruvate and participation of the latter in the reactions of the tricarboxylic acid cycle. The relatively better utilization of glucose for serine and glycine biosynthesis would be readily explained by the assumption that the C_3 serine precursor is more closely related to an intermediate of glucose catabolism, such as a triose or glyceric acid, than to pyruvate. The equal utilization of uniformly labelled [^{14}C]glucose and [1-^{14}C]glucose for serine biosynthesis further suggests that this precursor arises by the reactions of the glycolytic pathway, rather than by the oxidative metabolism of glucose to pentose in which carbon atom 1 is lost as carbon dioxide.

Both serine and glycine isolated after feeding uniformly labelled [^{14}C]glucose contained essentially equal amounts of ^{14}C in each carbon atom. After feeding [1-^{14}C]glucose 55–65 % of the total radioactivity in serine was located in the β-position, indicating preferential synthesis of this carbon atom from $C_{(1)}$ of glucose. The radioactivity in the other positions of the serine molecule in this experiment is probably due to redistribution of ^{14}C as a result of the subsequent metabolism of newly formed β-labelled serine. Thus, it has been shown (Elwyn & Sprinson, 1950*a*; Arnstein, 1951) that L-[β-^{14}C]serine gives rise to a limited but appreciable extent to labelled glycine, which in turn would be converted into serine labelled in the α- and carboxyl-carbon atoms. In other experiments, however, the β-carbon atom of serine isolated from rats given L-[β-^{14}C]serine for 2 days contained 99 % of the total radioactivity, and the glycine was almost inactive (Elwyn & Sprinson, 1954). It would appear, therefore, that the extent of redistribution of ^{14}C from [β-^{14}C]serine into other positions is somewhat variable, possibly depending on the animal's requirement for glycine. On the whole, therefore, the equal utilization of uniformly labelled [^{14}C]glucose and [1-^{14}C]glucose and the distribution of ^{14}C in the serine molecule suggests the utilization of carbon atoms 1–3 of glucose after catabolism to an intermediate of the glycolytic pathway.

Incorporation of radioactivity from [^{14}C]glucose into alanine as an indication of the pathway of glucose metabolism

The oxidative pathway of glucose metabolism to pentose results in the preferential loss of carbon atom 1 of glucose as carbon dioxide, and a comparison of the conversion of [^{14}C]glucose, labelled in different positions, e.g. $C_{(1)}$ and $C_{(6)}$, into carbon dioxide has been used to estimate the respective contributions of the glycolytic and oxidative pathways (Bloom, Stetten & Stetten, 1953; Bloom & Stetten, 1955). Similarly, pyruvate can arise from carbon atoms 1–3 of glucose only by glycolysis, whilst carbon atoms 4–6 are converted into pyruvate both by glycolysis and by the oxidative pathway. Since alanine is metabolically closely related to pyruvate, the incorporation of radioactivity into this amino acid from glucose labelled in various carbon atoms may also be used as an indication of the relative importance of these two pathways of glucose metabolism.

In the present experiments there was no detectable difference in the radioactivity of alanine isolated from the crude proteins of the internal organs of rats fed on either [1-^{14}C]glucose or uniformly labelled [^{14}C]glucose, indicating that, in agreement with the results on the oxidation of labelled glucose to carbon dioxide (Bloom *et al.* 1953), the oxidative metabolism of glucose to pyruvate was quantitatively unimportant in the intact rat. One important difference, however, between the present experiments, which are similar to those recently used to estimate the pathways of glucose catabolism in yeast (Blumenthal, Lewis & Weinhouse, 1954), and those based on the conversion of glucose into carbon dioxide, is that only pentose which is catabolized to pyruvate is estimated by the former method, whilst the latter method includes pentose utilized for synthetic reactions such as nucleotide synthesis. It is clear, therefore, that the two methods give minimum and maximum values respectively for the metabolism of glucose by the oxidative pathway.

The absence of any significant contribution of the oxidative pathway to the conversion of glucose into pyruvate in the intact animal suggests that it represents essentially a mechanism for the biosynthesis of the ribose moiety of nucleotides, but is of little importance for the catabolism of glucose.

Conversion of glucose into glutamic and aspartic acid

The greater dilutions of radioactivity during the conversion of uniformly labelled glucose into glutamic acid and aspartic acid compared with those in both [1-^{14}C]glucose experiments are probably due to the loss of radioactivity originally present in carbon atoms 3 and 4 during the formation of citrate from pyruvate, in which the carboxyl carbon of pyruvate is converted into carbon dioxide. In the [1-^{14}C]glucose experiments no radioactivity is lost at this stage. The radioactivity of the newly synthesized citrate would thus be equal to that present originally in $C_{(1)}$, $C_{(2)}$, $C_{(5)}$ and $C_{(6)}$ of the glucose. Further catabolism of citrate by the reactions of the tricarboxylic acid cycle would result in similar labelling of oxoglutarate and oxaloacetate, and hence glutamic acid and aspartic acid. It is noteworthy that both aspartic acid and glutamic acid show similar differences in radioactivity, whilst the radioactivity of alanine is the same in the experiments with [1-^{14}C] and uniformly labelled [^{14}C]glucose. It may be deduced from these results that carbon dioxide fixation leading to the direct synthesis of oxaloacetate from pyruvate, which would result in the incorporation of similar radioactivities into alanine and aspartic acid, is relatively unimportant under the conditions of these *in vivo* experiments.

SUMMARY

1. Young rats have been fed on synthetic diets containing 0·59% of L-[β-^{14}C]alanine, 0·5% of uniformly labelled L-[^{14}C]alanine, 34% of D-[1-^{14}C]-glucose or 34% of uniformly labelled D-[^{14}C]glucose for periods of 13–20 days under defined conditions in the presence and absence of dietary pteroylglutamic acid and vitamin B_{12}.

2. At the end of the experimental feeding period, alanine, aspartic acid, glutamic acid, serine and glycine were isolated from the protein of the mixed internal organs and their radioactivity was determined.

3. When uniformly labelled glucose was fed all the isolated amino acids had similar radioactivities. Degradation of the serine and glycine showed that the distribution of ^{14}C in these amino acids was essentially uniform. [1-^{14}C]Glucose gave rise to similar radioactivities in the isolated alanine and serine; the serine contained 55–65% of the total radioactivity in the β-carbon atom. When labelled alanine was fed serine and glycine were much less radioactive than the isolated alanine, aspartic acid and glutamic acid.

4. Deficiency of pteroylglutamic acid, but not of vitamin B_{12}, specifically reduced the incorporation of radioactivity from labelled alanine or glucose into glycine, whilst it had no effect on the radioactivity of serine.

5. It is concluded that serine is an important intermediate in glycine biosynthesis but that serine biosynthesis from non-nitrogenous sources does not require pteroylglutamic acid or the prior formation of glycine.

6. Serine is synthesized from a non-nitrogenous C_3 precursor which is more closely related to an intermediate of the glycolytic pathway of glucose catabolism, possibly a triose or glyceric acid, than to pyruvate.

7. The conversion of glucose, labelled in $C_{(1)}$ or in all carbon atoms, into alanine has been used to calculate the relative contribution of the glycolytic and oxidative pathways of glucose catabolism. In the intact rat none of the pyruvate appears to arise by the oxidative catabolism of glucose.

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The Effects of Some Inhibitors of Oxidative Phosphorylation on the Morphology and Enzymic Activities of Mitochondria

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It has been shown in previous experiments that uncoupling of oxidative phosphorylation occurs in mitochondria isolated from fatty livers. The same type of enzymic damage has been found to occur in mitochondria from normal livers after treatment with distilled water (Harman & Feigelson, 1952; Dianzani, 1954). Since both water-treated mitochondria and mitochondria from fatty livers are swollen and have a spherical form, it seems probable that this uncoupling of oxidative phosphorylation is intimately connected with the morphological changes. Since adenosine triphosphate (ATP) is necessary for the activation of fatty acids (Lipmann, Jones, Black & Flynn, 1952), the hypothesis that the accumulation of fat within the cell is the result of the uncoupling of the oxidative phosphorylation has been advanced (Dianzani, 1954). The mechanism of the uncoupling of oxidative phosphorylation occurring in both water-treated and 'fatty' mitochondria is not completely clear, but it seems very likely that the synthetic reaction $ADP + \sim P \rightleftharpoons ATP$ is affected (Dianzani, 1954).

Many substances have been reported to uncouple oxidative phosphorylation *in vitro*. 2:4-Dinitrophenol (DNP) and some compounds commonly used as histological stains are particularly active (Judah & Williams-Ashman, 1951; Lardy & Wellman, 1952; Potter, Siekevitz & Simonson, 1953; Chappell & Perry, 1953). The mechanism of

the action of DNP has been investigated by many authors. DNP has been found to increase the adenosine triphosphatase (ATPase) activity of mitochondria and also the activity of soluble ATPase (Lardy & Wellman, 1952).

The effect *in vivo* of the substances which uncouple oxidative phosphorylation *in vitro* has not yet been investigated. The effects of DNP and of five histological stains [methylene blue (MB), brilliant cresyl blue (BCB), thionine (Th), Janus Green B (JG) and phenosafranine (PS)] on the morphology and oxidative phosphorylation of mitochondria isolated from different organs have been studied, and the results are described in this paper. Particular attention was devoted to the study of the morphology of liver mitochondria and to the lipid content of whole liver.

MATERIAL AND METHODS

Mitochondrial preparations. Albino rats from an inbred strain, weighing 120–150 g., and albino mice from Strong A strain, weighing 20–22 g., were used. The animals were fed on a standard diet. They were killed by decapitation and the organs (liver, kidney, brain, heart and skeletal muscle) were immediately dissected, weighed and transferred to the cold room at 2°. Homogenization was made in a Potter–Elvehjem apparatus with a Lucite (polymethyl methacrylate, du Pont de Nemours) pestle, 10% (w/v) homogenates being prepared in 0·25 M sucrose. 0·88 M Sucrose was used in a few instances. With brain, only the cortex was used. Differential centrifuging was done in a Servall SS-1 Type Angle Centrifuge in the cold room. Tissue debris, undamaged cells, nuclei and some mitochondria (and also myofibrils in the case of heart and skeletal muscle) were discarded by centrifuging at 1000 *g* for 10 min. Mitochondria were then sedimented at 12 000 *g* for 30 min. when 0·25 M sucrose was used as suspension medium and at 22 000 *g* for 30 min. when 0·88 M sucrose was employed. Mitochondria were then washed twice with sucrose. The purity and morphology of the mitochondrial preparations were studied in a Zeiss phase-contrast microscope. Brain preparations were never pure, a certain amount of myelin material being always present. As the use of more elaborate purification procedures often produced damage to mitochondria, the use of non-purified sediments was preferred.

Oxidative phosphorylation. This was determined as described elsewhere (Dianzani, 1954), with 30 μmoles of α-oxoglutarate as substrate. The reaction mixture contained: 0·02 M-Na_2HPO_4–KH_2PO_4, pH 7·4; 0·001 M adenosine monophosphate (AMP); 0·0067 M-$MgSO_4$; 0·025 M-KCl; 10^{-5} M cytochrome *c*; 0·026 M fructose; 0·013 M-KF, 0·3 ml. hexokinase solution and 0·5 ml. of the mitochondrial suspension (corresponding to 100 mg. of fresh tissue). The final volume was 3 ml. KOH (30%, w/v, 0·2 ml.) was placed in the centre well of the Warburg flasks; temp., 25°. The O_2 uptake was measured for 10 min.; after this time, 0·5 ml. of 40% (w/v) trichloroacetic acid (TCA) was added from the side arm to stop the reaction.

Inorganic orthophosphate. This was determined in the protein-free filtrate according to Fiske & Subbarow (1925), with 40% (w/v) $SnCl_2$ dissolved in conc. HCl as a reducing agent. Readings of the optical density were made at 730 mμ. in a Beckman Model DU spectrophotometer.

Phosphorylated intermediates. In some experiments, the concentrations of certain phosphorylated intermediates in the protein-free filtrate were determined. The fractionation was made according to LePage (1951), by treatment with barium acetate at pH 8·2. The fraction giving insoluble barium salts was analysed for acid-labile P (increase of inorganic P after hydrolysis with N-HCl at 100° for 8 min.) and for ribose. In the experimental conditions used in this work, acid-labile P represents the inorganic P released from ATP + ADP. As ATP releases two phosphate groups/unit of ribose, and ADP only one phosphate group/unit of ribose, the relative concentrations of ATP and ADP may be deduced from the value of the molar quotient acid-labile P/ribose. The ethanol-insoluble fraction of the soluble barium salts was analysed for AMP and for fructose 6-phosphate. The concentration of AMP was determined by measuring the optical density at 260 mμ. after dissolving in acid, neutralization and dilution. Fructose 6-phosphate concentration was calculated from fructose determinations, made according to Roe (1934).

Hexokinase. This was prepared from wheat germ, and its activity was determined as described by Saltman (1953). A large excess of the enzyme was used in the phosphorylation experiments.

Measurement of enzyme activities. ATPase activity was determined in most experiments according to the method of Dubois & Potter (1943), with 0·067 M borate buffer, pH 6·9, 0·01 M ATP (Na salt) and 0·0067 M-$MgSO_4$. In other experiments, in which the tonicity of the reaction medium was important, the incubation medium had a different composition; this will be shown in each particular case.

Alkaline phosphatase activity was determined at pH 9·2, according to the method of Nguyen Van Thoai, Roche & Roger (1947), with 0·2 M sodium β-glycerophosphate as substrate and 0·0067 M-$MgSO_4$ as activator. Acid phosphatase was determined at pH 5·2, with 0·067 M veronal–HCl buffer, and 0·2 M sodium β-glycerophosphate. Inorganic pyrophosphatase was determined with 0·067 M borate buffer, pH 7·4, 0·0067 M-$MgSO_4$ and 0·01 M sodium pyrophosphate.

5′-Nucleotidase was determined by measuring the release of inorganic orthophosphate from 0·01 M AMP, with 0·067 M borate buffer, pH 7·4, and 0·0067 M-$MgSO_4$. In all phosphatase determinations, incubation was at 38° for 20 min., with 0·1 ml. of a 10% suspension of mitochondria in 0·25 M sucrose. In every case the enzymic reaction was stopped by addition of an equal volume of 40% (w/v) TCA.

Myokinase activity was determined according to Kielley & Kielley (1951), with a reaction mixture containing 0·05 M histidine, 0·005 M-$MgCl_2$, 0·04 M-KCl, 0·025 M glucose, 0·0007 M ATP, 0·1 ml. hexokinase, 0·1 ml. of adenylic acid deaminase, 0·1 ml. of the mitochondrial suspension and water to 3 ml. The mixture was pre-incubated at 27° for 10 min. with hexokinase and adenylic acid deaminase; the mitochondrial suspension was then added and the incubation at 27° was continued for 5 min. The reaction was stopped by addition of an equal volume of 10% (w/v) TCA. The decrease of the optical density at 260 mμ. occurring in these conditions was taken as a measure of myokinase activity. Adenylic acid deaminase was the preparation A of Kalckar (1947) from rabbit muscle;

0·1 ml. of this preparation was enough to destroy in 5 min. at 27° 40% of added AMP, when the concentration of this was 600 μg./ml. The enzyme was stored at −22° for 2 months without loss of activity.

Nitrogen. This was determined by a micro-Kjeldahl technique.

Total lipid content of the liver. This was determined by weighing the dry powder of the organ before and after extraction with ether for 3–4 hr. in a Kumagawa apparatus. Drying of the liver to constant weight was done in an oven at 85°.

Specimens for histological examination were prepared from each liver. Staining of fat was made with Sudan III; glycogen was stained by the method of Best (1906).

Reagents. The following substances were used as uncouplers of oxidative phosphorylation: DNP, methylene blue (3:7-bisdimethylaminophenothiazine chloride), thionine (3:7-diaminophenothiazine chloride), brilliant cresyl blue (3-amino-7-diethylamino-8-methyl-phenoxazine), Janus Green B (5-chloro-3-diethylamino-7-*p*-dimethylaminophenylazo-5-phenylphenazine) and phenosafranine, (2:8-diamino-10-hydroxy-10-phenylphenazine). DNP, JG and MB were products of the firm E. Merck, Darmstadt; BCB was furnished by Ciba Aktiengesellschaft, Basel; PS was a product of Eastman Kodak Co., Rochester 3, New York, and Th was a product of the firm G. Grübler Co., Leipzig.

ATP (sodium salt) was prepared from the dibarium salt (Schwarz Lab. Inc., New York 17, N.Y.), after solution in 0·1 N-HCl, and precipitation of barium with the calculated amount of H_2SO_4. AMP (sodium salt) was prepared by neutralization of the acid (Schwarz Lab. Inc.).

Cytochrome *c* was prepared from horse heart by the method of Keilin & Hartree (1945). Its Fe content varied from 0·35 to 0·40% in different preparations. Fe determinations were made according to the method of Elvehjem (1930). All other substances used were commercial preparations.

Statistical evaluation of results. The values obtained were submitted to statistical analysis, the standard deviation and Student's *t* test being calculated for each average. Only differences with a *t* value corresponding to a probability $P<0{\cdot}05$ were accepted as significant.

RESULTS

Inhibition of oxidative phosphorylation in vitro

The extent of the inhibition of oxidative phosphorylation caused by DNP and by the histological stains used in this work has been investigated by many authors, and particularly by Judah & Williams-Ashman (1951). It has been shown that these substances act mainly by inhibiting the uptake of inorganic orthophosphate from the reaction medium, whereas the oxygen uptake is unmodified or increased. The extent of uncoupling of the oxidative phosphorylation caused by the substances used has been the object of a preliminary study during the present investigation.

Three different concentrations of each substance were used, and their effect was studied on mitochondria isolated from liver, kidney, brain, heart and skeletal muscle. Table 1 shows that a 10^{-4} M concentration of the substances used inhibits the oxidative phosphorylation by about 90% in mitochondria from most organs. The percentage inhibitions produced by concentrations of 10^{-5} M were, however, not identical in all enzyme preparations. The difference was particularly striking in the case of DNP, which produced 94% inhibition in mitochondria from skeletal muscle; the inhibition did not exceed 50% in mitochondria from other organs. All the substances used affected mainly the uptake of inorganic orthophosphate, while the oxygen uptake was practically unmodified.

The effects of these substances on the morphology of mitochondria have been studied. Mitochondria isolated from approximately 100 mg. of tissue with 0·88 M sucrose were suspended in a small volume of 0·88 M sucrose. Small amounts of the inhibitors of oxidative phosphorylation dissolved in 0·88 M sucrose were then added to the mitochondrial suspension, the final concentration of the inhibitors being 10^{-4} M. The mixture was set aside at room temperature; every 5 min. a sample of the suspension was removed and the morphology of the mitochondria was examined under the phase-contrast microscope. Mitochondria isolated from hypertonic sucrose homogenates were mainly rod-like. Their form did not show any modification after being kept for 30 min. at room temperature. In the presence of DNP or of the other inhibitors of oxidative phosphorylation, a rapid change of form from rod-like to spherical form occurred. Nearly all mitochondria were swollen 15 min. after the addition of the uncoupling agent. The change was particularly rapid with DNP. This fact agrees with the observation made by Loomis & Lipmann (1948).

Inhibition of oxidative phosphorylation in vivo

In other experiments, the influence of a single injection of the inhibitors of oxidative phosphorylation on mitochondria from different organs was studied. Albino rats were injected intraperitoneally with 3 mg. of the inhibitor. Their body temperature was measured at regular intervals before and after the injection of the substances. All animals showed an increase of body temperature of about 1–2° within 12 hr. They were killed 20 hr. after the injection, and mitochondria were isolated from their organs for the measurement of oxidative phosphorylation. Table 2 shows that all injected substances produced uncoupling of oxidative phosphorylation in mitochondria of liver, heart, kidney and skeletal muscle. P/O quotients very close to normal were, however, obtained with mitochondria isolated from brain.

Mitochondria isolated either with 0·88 M or with 0·25 M sucrose from organs in which uncoupling of oxidative phosphorylation had occurred exhibited a spherical form and appeared to be swollen in comparison with mitochondria from normal organs. It is clear from these experiments that the substances used are active also *in vivo*. As they produced fever, the hypothesis may be advanced that some relationship exists between the uncoupling of oxidative phosphorylation and the fever.

Production of fatty infiltration in the liver on treatment with inhibitors of oxidative phosphorylation

As uncoupling of oxidative phosphorylation occurs in fatty livers (Dianzani, 1954), the effect of prolonged treatment with the inhibitors of this enzymic reaction on the total lipid content of the liver was studied. All substances were injected into albino rats or mice intraperitoneally, using 0·2 mg. daily for mice and 2 mg. for rats, six injections

Table 1. *Inhibition of oxidative phosphorylation by three different concentrations of the inhibitors*

30 μmoles of α-oxoglutarate were used as substrate. The results represent the mean values obtained in five experiments $\pm$S.D.

Inhibitor	Source of mitochondria (rat tissue)	P:O ratios without inhibitor added	Decrease of P:O ratios in the presence of the inhibitors (%)		
			Concn. of inhibitor (M)		
			$0{\cdot}1 \times 10^{-4}$	$0{\cdot}5 \times 10^{-4}$	10^{-4}
DNP	Liver	3·9±0·2	50·5±4·6	81·6±4·0	96·3±2·4
	Kidney	3·6±0·1	43·7±4·6	73·9±9·3	98·2±1·0
	Heart	3·5±0·2	36·4±7·2	86·3±3·0	96·7±1·4
	Muscle	3·2±0·1	94·0±3·6	100	100
	Brain	3·3±0·1	43·5±6·5	63·1±0·9	91·9±5·3
PS	Liver	3·9±0·1	73·6±7·1	83·6±2·8	98·1±0·6
	Kidney	3·6±0·1	64·0±8·0	81·5±2·4	94·6±2·4
	Heart	3·4±0·2	45·4±5·8	63·8±11·1	100
	Muscle	3·2±0·3	30·5±5·6	68·2±11·5	93·0±4·0
	Brain	3·5±0·4	36·4±4·6	72·5±8·2	96·4±0·6
BCB	Liver	3·9±0·2	48·8±3·6	66·6±5·4	82·8±6·8
	Kidney	3·7±0·1	56·2±4·5	70·7±4·6	85·0±6·4
	Heart	3·3±0·1	38·6±0·9	68·1±4·4	83·3±5·0
	Muscle	3·4±0·2	29·6±8·4	52·0±4·0	66·3±5·9
	Brain	3·5±0·4	56·8±4·7	72·8±5·0	92·0±6·1
Th	Liver	3·7±0·1	88·9±6·5	92·9±6·4	98·5±3·7
	Kidney	3·5±0·1	78·8±7·5	86·9±7·5	91·5±6·0
	Heart	3·6±0·2	64·6±5·9	72·0±5·8	100
	Muscle	3·4±0·2	24·6±9·4	54·0±6·2	77·1±11·4
	Brain	3·6±0·2	56·4±0·2	58·6±4·5	80·4±5·0
JG	Liver	3·7±0·2	74·5±8·9	80·1±3·5	98·9±1·0
	Kidney	3·8±0·3	73·0±2·0	79·9±3·0	96·3±4·5
	Heart	3·5±0·2	63·0±4·0	75·7±5·0	91·1±4·6
	Muscle	3·5±0·4	43·9±6·4	69·3±5·0	97·1±2·0
	Brain	3·6±0·2	47·5±5·0	60·9±4·2	84·7±5·0
MB	Liver	3·9±0·1	50·3±2·9	60·7±5·2	88·8±3·4
	Kidney	3·7±0·3	31·7±1·6	46·6±2·0	66·1±4·6
	Heart	3·7±0·1	10·4±2·0	35·6±1·9	47·3±3·0
	Muscle	3·7±0·2	5·8±2·0	32·8±2·0	47·9±2·0
	Brain	3·6±0·2	34·6±2·6	51·9±2·2	68·9±2·4

Table 2. P:O *ratios in mitochondria isolated from different organs of rats injected with inhibitors of oxidative phosphorylation*

The amount of the inhibitor injected intraperitoneally was 3 mg. The results represent the mean P:O values $\pm$S.D.

Inhibitor	No. of expts.	P:O ratios in mitochondria isolated from				
		Liver	Kidney	Heart	Skeletal muscle	Brain
DNP	6	0·65±0·16	1·95±0·10	1·78±0·48	0·28±0·20	3·1±0·45
PS	3	1·20±0·34	1·10±0·26	1·23±0·30	1·40±0·52	2·70±0·15
BCB	3	0·96±0·15	1·80±0·34	1·56±0·27	0·73±0·11	2·93±0·08
Th	3	0·90±0·1	1·33±0·22	1·00±0·44	1·26±0·30	3·00±0·34
JG	3	1·46±0·32	2·00±0·10	1·86±0·41	1·56±0·06	3·00±0·26
MB	3	0·77±0·05	1·73±0·37	1·20±0·29	0·73±0·20	2·70±0·34

being made in all. The animals were killed 24 hr. after the last injection. Table 3 shows that the total lipid content of the livers of treated animals is markedly increased. Those treated with DNP form the only exception, the total lipid content of their livers being very close to the normal value. Histological specimens stained with Sudan III showed a high degree of fatty infiltration of the evident. In rats killed after 36 hr., glycogen was not present, but small droplets of fat became evident within the liver cells. The number of fat droplets was very significant in the liver cells of rats killed after 48–96 hr. Infiltration with glycogen became evident after 120 hr. and was substantial after 144 hr. At these stages the number of droplets of neutral fat decreased progressively. It is clear from this experiment that damage of lipid metabolism is present also in the cells of the liver of rats treated with DNP.

Table 3. *Total lipid content of the liver of mice treated intraperitoneally with inhibitors of oxidative phosphorylation*

Inhibitor	No. of expts.	Total lipids (mg./g. of fresh liver)
None	6	29·0±2·2
DNP	4	36·0±2·2
PS	5	68·0±3·6
BCB	4	58·0±3·4
Th	3	52·4±4·6
JG	3	48·6±2·6
MB	4	74·0±5·6

Mechanism of inhibition of oxidative phosphorylation produced by DNP and by other substances used

Two possible types of mechanism may have been responsible for the decreased uptake of inorganic orthophosphate from the reaction medium in oxidative phosphorylation: (i) a real decrease of the synthesis of ATP; (ii) an increased destruction

Table 4. *Influence of DNP and other substances on the phosphatase activities of liver mitochondria of normal rats*

The results represent mg. of inorganic orthophosphate liberated in 1 hr. by mitochondria isolated from 1 g. of liver, ±S.D. Concn. of the inhibitor, 10^{-4} M.

Enzyme	No. of expts.	Inhibitor added: None	DNP	PS	BCB	Th	JG	MB
Acid phosphatase	5	2·71±0·02	3·93±0·72	2·66±0·60	3·23±0·20	2·59±0·80	1·84±0·20	2·57±0·40
Alkaline phosphatase	5	2·80±0·43	3·90±0·21	2·55±0·10	4·42±0·80	2·38±0·60	2·94±1·30	2·75±0·30
Inorganic pyrophosphatase	4	7·00±0·40	6·40±0·50	6·90±0·20	6·20±0·30	7·10±0·50	6·40±0·60	6·30±0·21
5′-Nucleotidase	4	2·68±0·31	3·65±0·60	2·34±0·27	3·32±0·17	2·72±0·60	2·50±0·18	2·70±0·81
ATPase	5	8·50±0·31	24·0±0·80	9·90±0·22	16·40±0·60	8·80±0·63	7·81±0·82	7·60±0·50

centrolobular type in all treated animals, with the exception of those treated with DNP. Fatty infiltration was already evident 36–48 hr. after the first injection. Rats treated with DNP showed a high degree of infiltration of glycogen rather than of fat. A very similar observation has been made by Barnes (1953) on the livers of rats treated with 2:5-dinitro-*o*-cresol and by Fonnesu & Severi (1955) on those of rats treated with DNP. As the reason for the different behaviour of DNP from that of other uncouplers was not clear, some experiments were carried out in order to discover the first stages of modification occurring in the liver cells after the injection of DNP. For this purpose, 2 mg. of DNP were injected into rats daily and the animals were killed 12, 24, 36, 48, 96 and 144 hr. after the first injection. Histological specimens were prepared from the livers and these were stained both with Sudan III and by the same method as that used by Best (1906) for glycogen. In the first 12–24 hr. only a slight degree of swelling was observed in liver cells, neither fat nor glycogen being morphologically

of normally formed ATP or of other phosphorylating intermediates. For this reason, the influences of all the inhibitors on ATPase, 5′-nucleotidase, inorganic pyrophosphatase and on both acid- and alkaline-phosphatase activities of mitochondria were studied. Particular attention was devoted to the study of liver mitochondria. The influence of the inhibitors was studied *in vitro* as well as *in vivo*. Table 4 shows that only DNP and BCB, in 10^{-4} M concentration, produced any degree of stimulation of ATPase, 5′-nucleotidase and of both acid and alkaline phosphatases *in vitro*. Table 5 shows that all the inhibitors, when injected intraperitoneally in amounts of 2 mg. daily for 3 days, produced an increase of ATPase as well as of both acid and alkaline phosphatases. Since the liver of the animals contained an increased amount of fat in most cases, the possibility exists that the increase of enzymic activity results from the morphological modifications which occur in fatty livers (Dianzani, 1954; Berthet, Berthet, Appelmans & Duve, 1951).

The mechanism of ATPase stimulation produced by DNP has been extensively studied by Potter *et al.* (1953) and by Lardy & Wellman (1952). These authors reported that DNP is active on both fresh and pre-aged mitochondria. Pre-ageing consisted in incubating at 37° for 20 min. Lardy & Wellman (1952) observed that soluble ATPase was also stimulated by DNP.

Two types of mitochondrial preparation were used: the first was a suspension of liver mitochondria in 0·25 M sucrose; with the second the medium was the same, but the suspension was incubated at 28° for 30 min. It is known that ageing of mitochondria produces both damage to the membrane and activation of ATPase (Potter & Recknagel, 1951; Kielley & Kielley, 1951). In these experiments, the medium of Dubois & Potter (1943) was not used, in order to avoid as far as possible any osmotic damage to the mitochondria. 0·25 M Sucrose was used as a suspension medium in every case; all other added substances were also dissolved in 0·25 M sucrose. The reaction mixture contained 6 μmoles of ATP, 0·04 M-KCl, mitochondria from 10 mg. of liver, and, when present, 0·001 M-$CaCl_2$, 0·002 M-$MgCl_2$, 3×10^{-5} M DNP and 3×10^{-5} M BCB. Table 6 shows that the addition of Ca^{2+} or Mg^{2+} to fresh mitochondria produced marked activation of ATPase. When both cations were added at the same time, the degree of activation was markedly higher. This fact agrees with the results reported by other workers (Potter *et al.* 1953; Lardy & Wellman, 1952). DNP produced a marked activation when used alone, but the extent of the stimulation produced was decreased in the presence of Mg^{2+} or Ca^{2+}. The same phenomenon occurred with BCB. Simultaneous addition of DNP and BCB did not increase the stimulation produced by DNP alone. The influence of the inhibitors on ATPase was very different when mitochondria were previously incubated at 28° for 30 min. ATPase activity of these mitochondria, when determined without any activator, was higher than the activity of fresh mitochondria. The effect of the addition of Mg^{2+} was very much higher, while that of Ca^{2+} was slight. The use of Ca^{2+} and Mg^{2+} together did not increase the stimulation produced by Mg^{2+} alone. Stimulations by DNP and by BCB were lower than those which occurred with fresh mitochondria, but were consistent in every case. Addition of DNP or BCB did

Table 5. *Influence of intraperitoneal injection of DNP and of other substances on some phosphatase activities of rat-liver mitochondria*

The results are expressed similarly to those in Table 4. Into the rats was injected 2 mg. of DNP or other substance intraperitoneally for 3 days and the rats were killed 24 hr. after the last injection.

Enzyme	No. of expts.	Inhibitor injected: None	DNP	PS	BCB	Th	JG	MB
Acid phosphatase	5	2·71±0·02	3·28±0·09	3·36±0·12	3·29±0·11	3·00±0·11	3·20±0·10	3·94±0·04
Alkaline phosphatase	5	2·80±0·43	3·84±0·08	4·43±0·28	4·10±0·49	3·50±0·10	3·77±0·12	4·47±0·22
Inorganic pyrophosphatase	4	7·00±0·40	3·60±0·40	3·50±0·10	3·70±0·10	3·60±0·10	3·30±0·40	3·70±0·90
5′-Nucleotidase	4	2·68±0·30	2·90±0·01	2·80±0·02	3·20±0·03	3·00±0·02	3·10±0·03	2·89±0·30
ATPase	5	8·50±0·31	17·80±0·70	18·2±0·40	12·8±0·40	10·4±0·80	9·60±0·40	12·8±0·30

Table 6. *Effect of* Mg^{2+}, Ca^{2+}, *DNP and BCB on ATPase of rat-liver mitochondria*

Concn. of Mg^{2+} was 0·002 M, that of Ca^{2+} 0·001 M, those of DNP and of BCB were 3×10^{-5} M. The mitochondria used contained 0·06 mg. of N. The values are given as μmoles of inorganic orthophosphate liberated in 15 min. at 38°, ±S.D.

Substance added	Fresh mitochondria	Mitochondria aged at 28° for 30 min.
None	0·25±0·02	0·65±0·03
Mg^{2+}	0·40±0·02	1·94±0·04
Ca^{2+}	0·51±0·04	0·80±0·02
Ca^{2+} and Mg^{2+}	1·11±0·06	1·68±0·04
DNP	1·00±0·04	0·90±0·01
DNP and Mg^{2+}	0·90±0·03	1·84±0·02
DNP and Ca^{2+}	0·46±0·01	0·80±0·03
DNP, Ca^{2+} and Mg^{2+}	0·80±0·02	1·60±0·05
BCB	0·80±0·02	0·90±0·02
BCB and Mg^{2+}	0·41±0·02	1·35±0·04
BCB and Ca^{2+}	0·98±0·03	0·82±0·02
BCB, Ca^{2+} and Mg^{2+}	0·60±0·02	0·65±0·02
BCB, DNP, Ca^{2+} and Mg^{2+}	0·66±0·01	0·90±0·03

In order to study the mechanism of the stimulation of ATPase which is produced by BCB, experiments were made in which the percentage activation was studied with regard to (i) the concentration of inhibitor, (ii) the concentration of enzyme, (iii) the concentration of stimulating cations (Mg^{2+} and Ca^{2+}), (iv) the state of the mitochondrial membrane and (v) the behaviour of DNP under the same conditions. In a first group of experiments, the effect of BCB, DNP, Ca^{2+} and Mg^{2+} or of different mixtures of these substances on the ATPase activity of liver mitochondria was studied.

not increase the effect of Mg^{2+} or of Ca^{2+}. These results agree with those obtained for DNP, Ca^{2+} and Mg^{2+} by Lardy & Wellman (1952). It seems likely from these experiments that the stimulation of ATPase produced by these substances is not entirely a consequence of the modification of the form of mitochondria. Mg^{2+}, whose stimulating activity was maximal on aged mitochondria, tions of fresh mitochondria from rat liver. The results are shown in Fig. 1. The activation produced under these conditions by DNP was proportional to the amount of enzyme used, when this did not exceed that corresponding to 0·18 mg. of N. With larger amounts of the enzyme, the stimulation produced by DNP was proportionally lower. In the case of BCB, the increase of ATPase activity

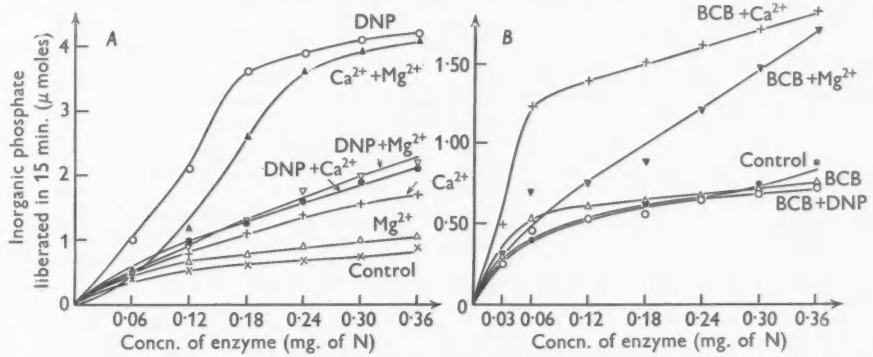

Fig. 1. Influence of the concentration of enzyme on the stimulation of ATPase activity by DNP, BCB, Mg^{2+} and Ca^{2+}. *A*, DNP; *B*, BCB. Mitochondria were isolated from rat liver. The concentration of DNP was 3×10^{-5} M, that of BCB 10^{-4} M, and those of Ca^{2+} and Mg^{2+} 0·0075 M. The final volume was 3 ml.

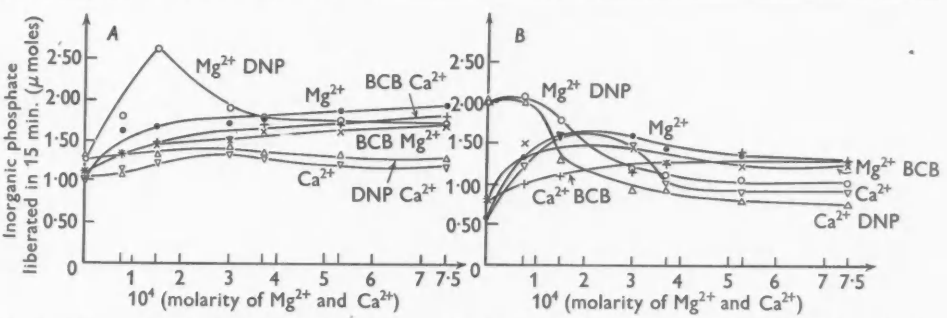

Fig. 2. Influence of concentration of Mg^{2+} and Ca^{2+} on the stimulation of ATPase by DNP and BCB. *A*, aged mitochondria; *B*, non-aged mitochondria. The concentration of DNP was 3×10^{-5} M, that of BCB 10^{-4} M. The *N* content of the mitochondria was 0·06 mg.

probably exerts its effect directly on the enzyme, while Ca^{2+}, which had only a slight influence on aged mitochondria, probably exerts its influence through a morphological modification of the particles. This was also the conclusion of Slater & Cleland (1953). The fact that Ca^{2+} and Mg^{2+} have a synergistic action on fresh mitochondria agrees with this interpretation.

In a second group of experiments, the influence of 3×10^{-5} M DNP, 10^{-4} M BCB, 0·0075 M-Ca^{2+} or 0·0075 M-Mg^{2+} was studied on different concentra- was smaller than that produced by DNP; when BCB was used together with Ca^{2+}, the stimulation of ATPase was proportionally higher for low concentrations of the enzyme than for high concentrations. The stimulation produced by Mg^{2+} alone was slight, but that found with the mixture of Ca^{2+} and Mg^{2+} increased progressively with the increase of the amount of the enzyme used.

In a third group of experiments, the influence of different concentrations of Ca^{2+} or Mg^{2+}, alone or in the presence of DNP or BCB, on ATPase activity

of both fresh and aged mitochondria was investigated. It is clear from Fig. 2 that the greatest stimulation was exerted, with fresh mitochondria, by 3×10^{-3}M-Mg^{2+}. With aged mitochondria, the degree of stimulation increased up to a concentration of $7{\cdot}5 \times 10^{-3}$M. This fact agrees with the idea that Mg^{2+} acts directly on the enzyme. For Ca^{2+}, increase of concentration up to 3×10^{-3}M produced an increase of the activation of ATPase on fresh as well as on aged mitochondria. The addition of BCB or of DNP decreased the effect of Ca^{2+} as well as that of Mg^{2+}. These results agree with similar observations made by Lardy & Wellman (1952) on DNP. Fig. 3 shows the influence of increasing concentrations of DNP and BCB in either the presence or the absence of 0·0075M-Mg^{2+}, on ATPase activity of both fresh and aged mitochondria. $0{\cdot}1 \times 10^{-4}$M DNP, which produced 60–70% inhibition of oxidative phosphorylation, strongly increased the ATPase activity of fresh mitochondria. In another group of experiments, the effect of DNP and of BCB on soluble ATPase, prepared from fresh mitochondria of rat liver according to the procedure of Lardy & Wellman (1952), was investigated (Table 7). It is clear from this table that Mg^{2+} produced activation of soluble ATPase, while Ca^{2+} was inactive. Both DNP and BCB exerted a strong stimulating activity. On the basis of these results, the action of Ca^{2+} seems to be mainly concerned with the damage of the mitochondrial membrane, whereas Mg^{2+}, DNP and BCB seem to act directly on the enzyme.

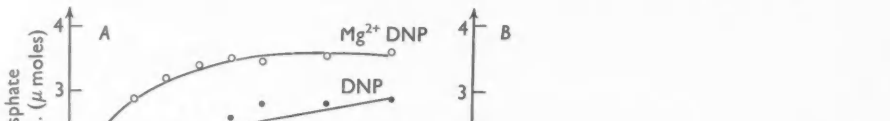

Fig. 3. Influence of concentration of BCB and DNP on the stimulation of ATPase activity of rat-liver mitochondria. *A*, aged mitochondria; *B*, non-aged mitochondria. The concentration of Mg^{2+} and of Ca^{2+}, when present, was 0·0075M. All the other experimental conditions were as in Fig. 2.

Table 7. *Influence of* Ca^{2+}, Mg^{2+}, *DNP and BCB on the soluble ATPase of rat-liver mitochondria*

The results represent μmoles of inorganic orthophosphate liberated in 10 min. at 38° by an amount of enzyme containing 0·378 mg. of N. Concentration of ATP, 0·01M.

Concn. of the substance tested (10^{-3}M)	Substance added			
	Ca^{2+}	Mg^{2+}	DNP	BCB
0	0·55	0·55	0·55	0·55
0·75	0·60	0·77	0·67	0·62
1·50	0·64	0·77	0·74	0·60
2·70	0·56	0·79	1·00	0·64
4·50	0·60	0·79	1·04	0·69

Myokinase

As the uncoupling of oxidative phosphorylation produced by the substances studied may be a consequence of a decrease in the formation of ADP through the myokinase reaction, the influence of these substances on myokinase was studied both *in vitro* and *in vivo*. Table 8 shows that, of all the substances used, only phenosafranine inhibits significantly myokinase activity of rat-liver mitochondria *in vitro* as well as *in vivo*. Since in the systems for

Table 8. *Influence of DNP and of other substances on myokinase activity of rat-liver mitochondria*

The results represent μmoles of ADP which disappeared from the reaction medium in 5 min. in the presence of the mitochondria isolated from 1 g. of liver. In the experiments *in vitro*, the concentration of the substance tested was 10^{-4}M. The results represent the mean of five experiments ±S.D.

Substance added	Expts. *in vitro*	Expts. *in vivo*
None	22·2±1·8	22·2±1·8
DNP	24·3±0·9	24·1±1·3
PS	12·9±1·7	16·4±0·4
BCB	24·0±0·9	22·5±1·3
Th	19·5±1·2	20·3±0·7
JG	18·9±0·4	21·6±1·2
MB	23·4±0·8	22·2±1·9

the determination of myokinase activity an inhibition of ADP splitting may be due to damage of hexokinase or of adenylic deaminase as well as to a real inhibition of myokinase, the influence of all the substances on hexokinase and on adenylic deaminase was studied. The results obtained were, however, completely negative.

Analysis of phosphorylated intermediates

The results of analysis of the phosphorylated intermediates that accumulate in the reaction medium under the conditions of oxidative phosphorylation are reported in Table 9. Sodium L-glutamate (30 μmoles) was used as a substrate instead of α-oxoglutarate in these experiments. It was found that in the presence of DNP the amount of AMP which disappeared from the reaction medium was decreased by only 34%, while P uptake decreased by about 87%. The sum of ATP and ADP (acid-labile P) decreased by about 52%, and the molar quotient acid-labile P/ribose decreased from 1·9 to 1·22. It is clear from these results that relative accumulation of ADP occurs in the reaction system. The mechanism of the uncoupling seems then to be related to inhibition of the reaction $ADP + \sim P \rightleftharpoons ATP$. Nearly the same type of modification in the distribution of phosphorylated intermediates occurred with MB, Th, JG and BCB. With PS the mechanism of inhibition of oxidative phosphorylation seems to be different from that occurring with other substances. In fact, the amount of AMP which disappeared from the reaction medium with PS was low, as also was the concentration of ADP. The molar quotient acid-labile P/ribose was 1·51. It appears probable then that with PS the inhibition of myokinase exerts a role in the inhibition of oxidative phosphorylation. It is also possible, however, that the synthesis of ATP from ADP + P is also directly affected. In fact, in some experiments, in which ADP was used instead of AMP as phosphate acceptor for the measurement of oxidative phosphorylation, very low P:O ratios were obtained in the presence of PS.

Table 9. *Analysis of phosphorylated intermediates contained in the reaction medium after* 10 *min. respiration in the system used for the determination of oxidative phosphorylation*

30 μmoles of glutamate were added as a substrate. The concentration of the inhibitors, when added, was 3×10^{-4} M. The results refer to mitochondria isolated from 100 mg. of liver.

Substance added	P disappearing (μatoms)	O taken up (μatoms)	P:O	P disappearing (μg.)	Acid-labile P (μg.)	Ribose (μg.)	Acid-labile P/ribose	ATP formed (μg.)	ADP formed (μg.)	AMP disappearing (μg.)	Fructose-6-phosphate formed (μg.)
None	19·0	6·5	2·9	584	118	300	1·90	581	85	460	2500
DNP	2·4	7·0	0·34	75	57	225	1·22	69	321	300	102
PS	4·7	5·0	0·90	144	31	100	1·51	28	35	50	600
MB	9·4	6·0	1·56	288	65	280	1·13	20	176	160	1080
None	16·1	5·4	2·6	432	108	280	1·88	518	89	450	1980
Th	0·9	1·2	0·7	28	20	70	1·39	42	84	105	60
JG	1·2	1·4	0·8	36	26	100	1·21	30	141	160	80
BCB	1·4	1·0	1·4	43	36	120	1·45	88	136	165	60
None	18·6	7·0	2·65	576	125	335	1·80	545	172	510	1800
DNP	1·4	7·5	0·20	45	36	145	1·20	39	198	160	80
PS	1·3	5·6	0·23	40	31	100	1·51	93	105	145	70
MB	6·4	6·0	1·06	200	62	250	1·20	67	341	331	1020
None	15·8	6·0	2·63	490	102	265	1·87	489	132	430	2700
Th	1·2	3·0	0·40	37	24	80	1·45	59	91	120	114
JG	1·2	3·0	0·40	37	28	112	1·20	30	154	158	82
BCB	1·5	4·2	0·36	47	42	140	1·45	107	158	220	45

DISCUSSION

The results obtained in the present investigation may be summarized as follows: (i) DNP, BCB, MB, Th, JG and PS, which uncouple oxidative phosphorylation *in vitro*, produce the same type of damage also *in vivo* of mitochondria isolated from liver, heart, kidney and skeletal muscle. The uncoupling of oxidative phosphorylation is in every case accompanied by the change of the form of mitochondria from rod-like to sphere-shaped and by consequent swelling. (ii) Prolonged treatment with many substances which uncouple oxidative phosphorylation produces a high degree of fat

infiltration of centrolobular type in the liver. Of all the substances used, only DNP produces accumulation of glycogen in the liver. In the first few hours after the beginning of the treatment with DNP, a deposition of fat was, however, present in the liver cells, before the beginning of glycogen infiltration. (iii) DNP and BCB produce activation of latent ATPase activity in mitochondria previously aged at 28° for 30 min. The same substances also produce stimulation *in vitro* of 5′-nucleotidase and of both alkaline and acid phosphatases. All the substances produce stimulation of ATPase, 5′-nucleotidase and acid and alkaline phosphatases *in vivo*. (iv) The mechanism of the action of BCB seems to be substantially similar to that of DNP with regard to ATPase activation. The activity of DNP is, however, larger than that of BCB. Both substances produce stimulation of soluble ATPase as well as of ATPase bound to the structure of the mitochondria. This fact agrees with the hypothesis that the activation of ATPase produced by DNP and BCB is not primarily concerned with a modification of the form of the mitochondria. It seems probable, on the contrary, that the morphological damage of these cell particles is a result of the activation of ATPase and of loss of ATP. (v) Myokinase activity of liver mitochondria is not modified by any of the substances, with the exception of PS, which inhibits this enzyme *in vitro* as well as *in vivo*.

It seems likely from these experiments that the substances exert their action on oxidative phosphorylation through different mechanisms. DNP and BCB, which stimulate ATPase activity of aged mitochondria and also stimulate soluble ATPase, act probably by such a mechanism. In fact, it seems evident that at least for DNP and BCB the activation of ATPase and the loss of ATP precede the morphological damage to the mitochondria. It is doubtful, however, if the activation of ATPase is entirely responsible for the uncoupling of oxidative phosphorylation. In fact, in the reaction mixture used for the phosphorylation measurements, the presence of 0·013 M-KF decreases the activity of ATPase. The hypothesis that the same enzyme produces both synthesis and destruction of ATP cannot be excluded; in this case the direction of the reaction would be reversed by DNP (Lardy & Wellman, 1952). The fact that MB, Th and JG, which, like DNP and BCB, uncouple oxidative phosphorylation, do not, however, strongly activate ATPase *in vitro*, indicates that the mechanism of action of these substances is probably different.

The mechanism of action of PS seems to be mainly concerned with the inhibition of myokinase, with consequent decrease of the amount of ADP available for the synthesis of ATP. The existence of a direct damage also to the enzyme which synthesizes ATP from ADP + P cannot, however, be definitely excluded.

The fact that all the substances produced uncoupling of oxidative phosphorylation *in vivo* seems noteworthy. In fact, all the histological dyes used in this work, as well as DNP, are well-known fever-producing agents. The inhibition of oxidative phosphorylation was particularly clear in mitochondria from skeletal muscles. DNP was more active than the other substances in this respect. Mitochondria isolated from the organs of animals treated with DNP and with other uncouplers were spherical, and this form corresponds to a higher oxidative activity (Harman, 1950; Dianzani, 1953). In addition, Green & Stoner (1950) have established that the oxygen uptake of the tissues is markedly decreased in animals treated with ATP. The presence of metabolic and morphological damage in skeletal muscle in fever has been described by many authors (Vernoni, 1954). In view of these findings, the hypothesis that the morphological modifications of mitochondria, and especially of those contained in skeletal muscle, may have an important role in the pathogenesis of fever should be given full consideration.

All the substances used produced accumulation of fat within the liver cells. This fact favours the hypothesis that uncoupling of oxidative phosphorylation is an important step in the pathogenesis of fatty liver degeneration. It is, however, not yet clear whether the uncoupling of oxidative phosphorylation is itself directly responsible for the fatty infiltration through a decrease of the activation of fatty acids or whether its action is mediated through other mechanisms concerned with the modification of the form of the mitochondria. A redistribution of cytochrome *c* (Dianzani & Viti, 1955) and of pyridine nucleotides (Dianzani, 1955) occurs in homogenates prepared from fatty livers, and this is probably due to the increased permeability of morphologically modified mitochondria. Accumulation of glycogen in the liver resulting from treatment with 2:5-dinitro-*o*-cresol was reported by Barnes (1953). Fonnesu & Severi (1955) have reported accumulation of glycogen in the liver of rats treated with DNP. The mechanism of glycogen accumulation is difficult to understand. It is interesting, however, to remember that treatment of rats with ATP introduced intraperitoneally produces glycogen depletion in the liver (Green & Stoner, 1950). It is therefore probable that the accumulation of glycogen is due to a deficiency of ATP. The reason why DNP produces mainly glycogenic infiltration, while other uncouplers produce fatty infiltration in the liver, is not clear. The difference in behaviour may be perhaps related to the extent of damage produced in skeletal

muscle, which is particularly great with DNP. Further experiments on these problems are in progress.

SUMMARY

1. The influence of 2:4-dinitrophenol, methylene blue, brilliant cresyl blue, thionine, phenosafranine and Janus Green B on the oxidative phosphorylation of mitochondria isolated from liver, kidney, brain, heart and skeletal muscle of rats has been studied *in vitro* as well as *in vivo*.

2. All the substances used produced a high degree of inhibition of oxidative phosphorylation both *in vitro* and *in vivo*. In every case, uncoupling of oxidative phosphorylation was accompanied by a change of form of the mitochondria from rod-like to spherical and by swelling.

3. 2:4-Dinitrophenol and brilliant cresyl blue produce activation of adenosinetriphosphatase of liver mitochondria *in vitro*. 5′-Nucleotidase and both acid and alkaline phosphatases are also stimulated. All the substances used stimulated adenosinetriphosphatase, 5′-nucleotidase and both acid and alkaline phosphatases *in vivo*.

4. 2:4-Dinitrophenol and brilliant cresyl blue stimulate adenosinetriphosphatase activity of fresh as well as of aged mitochondria. Also soluble adenosinetriphosphatase is activated by these substances.

5. Phenosafranine strongly inhibits myokinase of liver mitochondria, both *in vitro* and *in vivo*.

6. Prolonged treatment of rats with the substances produces fatty infiltration of the liver. 2:4-Dinitrophenol produces glycogen accumulation instead of fatty infiltration.

7. The significance of the phenomena is discussed.

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Studies on the Metabolism of the Protozoa

7. COMPARATIVE CARBOHYDRATE METABOLISM OF ELEVEN SPECIES OF TRYPANOSOME*

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From the numerous papers on the carbohydrate metabolism of trypanosomes which have appeared during the past twenty years, it has become obvious that, with the possible exception of *Trypanosoma cruzi* (Brand, Tobie, Kissling & Adams, 1949) and the plant trypanosome *Strigomonas oncopelti* (Ryley, 1955), cellular motility depends on a supply of extracellular monosaccharide, which is incompletely broken down to a mixture of organic acids; much of this work is admirably summarized by Brand (1951). Trypanosomes of the *brucei-evansi* group [for classification

* Part 6: Manners & Ryley (1955).

of trypanosomes see Hoare (1949)] seem to carry glucose breakdown no further than the pyruvic acid stage, and *T. lewisi* and *S. oncopelti* (Ryley, 1951, 1955) are able to carboxylate and decarboxylate pyruvic acid with the eventual production of succinic acid and acetic acid or ethanol. There is likewise a gradation in cyanide sensitivity between the *brucei-evansi* group and other species (Brand & Johnson, 1947). In the present study an opportunity has been taken to compare the extent of glucose breakdown under both aerobic and anaerobic conditions by eleven species of trypanosome, and to supplement observations on respiratory sensitivity to cyanide in a number of these species by investigations of the cytochrome spectra and the presence in homogenates of oxidative enzymes.

MATERIALS AND METHODS

Organisms and methods of culture. The trypanosomes used in the present studies were strains maintained in this Laboratory for chemotherapeutic work. The cultural forms of *T. cruzi* were grown in 250 ml. flasks, using Chang's (1947) diphasic medium; organisms were harvested after 14 days' growth at 26°, six flasks usually providing sufficient material for one experiment. The blood-stream forms of *T. cruzi* were obtained from batches of about 200 infected mice (20–30 g.) which were bled when the trypanosome population in the peripheral blood was at its maximum, usually 8–10 days after inoculation. *T. gambiense* was obtained from batches of 6–10 fully grown guinea pigs which were examined daily until the animals seemed heavily infected, a variable period of about 2–4 weeks after inoculation. *T. vivax* and *T. congolense* were grown in batches of 30–50 fully grown rats (discarded breeding stock), which were bled 4–5 days after inoculation; in these infections the trypanosome population increases to a maximum at about this time, after which either the animal dies or the trypanosomes gradually disappear from the blood, the infection relapsing at a later date. *T. rhodesiense*, *T. brucei*, *T. equinum*, *T. equiperdum* and *T. evansi* were obtained from batches of 10–15 fully grown rats, which were bled 3 days after infection, at a time when the trypanosome population was near the maximum compatible with the life of the host. *T. lewisi* was obtained from batches of about 30 rats (150 g.), which were bled 10 days after infection when the parasites were in the 'adult phase'.

Antisera to mouse and rat red cells. These were prepared in rabbits as described by Moulder (1948), freeze-dried, and stored at room temperature for periods up to 1 year; they were reconstituted with distilled water immediately before use.

Harvesting of organisms and preparation of suspensions. Infected animals were chloroformed and bled from the heart by means of a syringe, the blood being mixed with an equal volume of a solution containing 0·8% (w/v) of NaCl and 1% (w/v) of sodium citrate. In experiments in which citric acid determinations were made, infected blood was mixed with a little heparin instead of citrated saline. Trypanosomes were separated from the blood by differential centrifuging at room temperature, using an International SB size 1 centrifuge. The blood was centrifuged for 5 min. at 3000 rev./min., the supernatant was discarded and the trypanosomes which separated as a white layer above the red cells were removed as completely as possible by means of a Pasteur pipette into Ringer-bicarbonate or Ringer-phosphate solution (Ryley, 1955) containing 0·015 M-glucose. The trypanosome suspension was again centrifuged, the upper layer being collected and the small lower layer of contaminating red cells rejected. This process was repeated until the preparation was free from blood elements. In very heavy blood infections, the initial trypanosome layer was thick, and two centrifugings sufficed to obtain a clean preparation of parasites. With *T. lewisi*, *T. cruzi* and *T. congolense*, the infections never became very heavy, and the trypanosome layer on centrifuging was quite thin. In order to facilitate removal of red cells in these cases, the preparation after two centrifugings was diluted to give an approximately 10% (v/v) suspension with respect to the contaminating red cells, and excess of antiserum (having a titre usually of 1/20) was added, the mixture being incubated for 5 min. at 37°. The agglutinated red cells were removed by a brief (30 sec.; 500 rev./min.) centrifuging, after which the trypanosomes were spun down (5 min.; 3000 rev./min.) and washed free of serum, using Ringer-bicarbonate or Ringer-phosphate solution as required in the final preparation.

Metabolic experiments. Spectroscopic observations, manometric experiments and the subsequent estimation of metabolites were carried out as described for *S. oncopelti* by Ryley (1955); in addition, citric acid was estimated by the method of Ettinger, Goldbaum & Smith (1952).

RESULTS

The motility of most species of trypanosome depends on a supply of extracellular glucose or other utilizable carbohydrate. With the *brucei–evansi* group of trypanosomes, loss of motility on the removal of substrate coincides with a loss of respiratory activity, and is soon followed by cellular disintegration; an intact cellular structure in these organisms necessitates, among other things, a constant supply of utilizable carbohydrate. When isolated in inorganic media and supplied with glucose, these trypanosomes respire and maintain motility for relatively short periods of time. Such processes can be somewhat prolonged by the addition of serum or other protein to the medium, but, even so, conditions are far from ideal. In the present studies, protein-free suspending media have been used, combined with short-term experiments.

Other trypanosomes are not so dependent on a supply of extracellular glucose, and are more resistant to experimental manipulation *in vitro*. Thus *T. lewisi* (Ryley, 1953) can survive for 1–2 hr. in the absence of glucose, respiring with a Q_{O_2} (μl. of O_2 consumed/mg. dry wt./hr.) of 4·5 and R.Q. of 0·80; in the presence of glucose, the Q_{O_2} is increased to about 68, and the flagellates will respire at a fairly constant rate over a period of several hours. *S. oncopelti* (Ryley, 1955) has an

endogenous Q_{O_2} of 17·7 (30°) and R.Q. of 0·9; this respiratory activity is sufficient to support full motility over a period of many hours. Brand *et al.* (1949), in experiments using whole blood containing *T. cruzi*, were unable to detect any glucose utilization attributable to the parasites. In the present paper, some preliminary experiments carried out with *T. cruzi* will be reported before the carbohydrate metabolism is compared with that of the other trypanosomes.

Experiments with Trypanosoma cruzi

Cultural forms. These experiments were carried out at 30°. When suspended in a Ringer-bicarbonate medium in the presence of a gas phase of air containing 5% (v/v) of CO_2 and studied by the second method of Dickens & Šimer (1931), the flagellates were found to respire with a Q_{O_2} of 14·5 and an R.Q. of 0·77. The addition of glucose (0·015 M) caused an increase in respiratory rate (Q_{O_2} 21) and in R.Q. (1·00); respiration under these conditions continues for many hours at a steady rate. Respiration leads to an accumulation of organic acids in the presence, but not in the absence, of glucose.

One preparation of flagellates (45 mg. dry wt. in vol. of 6 ml.) was allowed to respire in the absence of glucose for a period of 210 min. During this time, 47·8 μmoles of oxygen were utilized (Q_{O_2} 7·0; R.Q. 0·77). There was no net acid production as estimated by bicarbonate disappearance, but analysis of the metabolic solution showed the formation of 7·1 μmoles of volatile acid and 19·8 μmoles of ammonia (by nesslerization); no glycogen could be detected in the cells.

Blood-stream forms. These experiments were carried out at 37°. A preparation of flagellates in Ringer phosphate (4·7 mg. dry wt. in 2·0 ml.) respired at a steady rate over a period of 4 hr. with a Q_{O_2} of 15·0; the addition of glucose (0·015 M) increased the Q_{O_2} to 31·6. Experiments in a Ringer-bicarbonate medium, using the Dickens & Šimer technique, gave R.Q. values of 0·77 in the absence and 0·92 in the presence of glucose. In the absence of glucose, respiration was not accompanied by bicarbonate breakdown, although acid was produced when glucose was present.

Glucose degradation by various trypanosomes

A series of experiments have been carried out in which different species of trypanosome, suspended in a Ringer-bicarbonate medium in the presence of 5% of CO_2 (v/v) in the gas phase, were allowed to degrade glucose under both aerobic and anaerobic conditions. Glucose was present in most cases all the time during preparation of the trypanosome suspensions. Glucose and its various degradation products were estimated before and after a period of incubation, which for the less robust organisms was kept as short as possible. Representative experiments of this series are summarized in Table 1. The table, besides including new data on nine species of trypanosome, includes for the purpose of comparison some previously published data on *T. lewisi* and *S. oncopelti* (Ryley, 1951, 1955). Some of the metabolites were determined at the lower limit of sensitivity of the methods employed, and are possibly of doubtful significance; those which are considered to be the main end-products of glucose degradation are indicated in heavier type. Citric acid estimations have been carried out in the case of six species; although only very small amounts have been detected, sufficient was produced to be readily measurable by the sensitive method of estimation used. Surprisingly enough, the greatest yields of citric acid were found with *T. rhodesiense*; substantially the same yields were obtained from trypanosomes suspended in plasma as in Ringer-bicarbonate solution. Apart from the two forms of *T. cruzi*, the estimated metabolites account for the major part of the glucose degraded. In addition to these results, no evidence has been found for the production of formic acid by *T. lewisi* or *T. cruzi*, of hydrogen by *T. lewisi*, *T. cruzi* or *S. oncopelti*, or of oxalic acid by *T. lewisi*.

Respiratory inhibition by cyanide and iodoacetate

The sensitivity of the respiration of six species of trypanosome to cyanide and iodoacetate has been determined in a limited number of experiments; the results are summarized in Table 2. For these determinations, trypanosomes have been used as suspensions in Ringer-phosphate–glucose or in whole blood. From the table it can be seen that the degree of inhibitions observed are similar for the two suspending media used apart from the case of *T. lewisi*, where respiration, although it is quite sensitive to iodoacetate in inorganic media, is very little affected in whole blood.

Cytochrome systems

From the cyanide-inhibition studies reported in Table 2, it would be expected that *T. lewisi*, *T. cruzi* and *S. oncopelti* should contain cytochrome pigments, whereas *T. vivax* and *T. rhodesiense* might not have these systems. The cytochrome systems of *T. lewisi* and *S. oncopelti* have already been described (Ryley, 1951, 1955). Spectroscopic examination of preparations of the cultural forms of *T. cruzi* reduced with sodium dithionite ($Na_2S_2O_4$) showed: (*a*) an intense band at 553–565 mμ.; (*b*) very faint β-bands at 525–535 mμ.; (*c*) a feeble band at 608 mμ. due to cytochrome *a*.

Table 1. *Metabolic products formed during aerobic and anaerobic glucose breakdown by eleven species of trypanosomes*

Aerobic experiments carried out in Dickens & Šimer manometer flasks with a Ringer-bicarbonate–glucose medium and a gas phase of CO_2 + air (5:95); anaerobic experiments carried out in Warburg flasks using a Ringer-bicarbonate–glucose medium with a gas phase of $CO_2 + N_2$ (5:95). Fermentations carried out at 37° unless otherwise indicated, being stopped after time shown by tipping in acid from a side bulb. Table indicates the total amount of glucose disappearing during the course of the fermentation and the amounts of metabolites recovered expressed in terms of moles of metabolite/mole of glucose disappearing. From this, carbon, acid and redox balances have been calculated, the last by the method of Johnson, Peterson & Fred (1931); the substrate glucose has a redox value of 0. Total cellular dry weight and volume of fermentation systems as indicated. Most of the values for *T. lewisi* taken from Ryley (1951) and for *S. oncopelti* from Ryley (1955).

	T. cruzi		*T. cruzi* (cultural; 30°)		*T. lewisi*		*S. oncopelti* (30°)		*T. congolense*		*T. vivax*	
	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2	CO_2/O_2	CO_2/N_2	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2
Duration of experiment (min.)	145	135	116	116	180	180	20	20	90	90	33	33
Dry wt./vol. (mg./ml.)	10·2/4·0	11·5/4·0	41·1/6·0	41·1/6·0	29·9/15·0	29·9/15·0	42·6/6·0	42·6/6·0	11·5/6·0	11·5/6·0	28·8/6·0	28·8/6·0
Glucose used (μmoles)	15·40	27·35	19·35	26·00	81·70	72·20	39·35	47·60	27·20	30·55	21·75	15·50
R.Q.	0·92	—	1·05	—	0·97*	—	2·50	—	1·01	—	0·72	—
Acid (bicarbonate)	1·40	2·76	1·17	2·79	1·19	2·50	1·01	1·08	2·22	2·34	2·53	1·61
O_2	3·60	—	2·49	—	3·17*	—	0·38	—	0·50	—	1·52	—
CO_2	**3·31**	**−0·53**	**2·61**	**−0·46**	**3·06***	**−0·74**	**0·95**	**0·46**	**0·50**	−0·09	**1·10**	**0·11**
Glycerol	0·00	0·03	0·00	0·00	0·00*	0·00*	**0·32**	**0·16**	**0·36**	**0·63**	**0·18**	**0·63**
Ethanol	—	—	0·00*	0·00*	0·05	0·02	**0·91**	**0·58**	0·00*	0·00*	0·00*	0·00*
Lactic acid	**0·12**	**0·22**	0·00*	0·00*	**0·12**	**0·39**	0·01	0·02	0·04	0·03	**0·16**	**0·30**
Pyruvic acid	0·02	0·08	0·00*	0·00*	0·03	**0·19**	**0·28**	**0·21**	0·00	**0·11**	**0·95**	**0·40**
Acetic acid	**0·34***	**0·23***	**0·62**	**0·61**	**0·47**	**0·18**	0·05*	0·09*	**1·00**	**0·46**	**0·80**	0·07*
Succinic acid	**0·23**	**0·47**	**0·14**	**0·67**	**0·18**	**0·89**	**0·22**	**0·43**	**0·49**	**0·70**	0·00	0·00
Citric acid	—	—	0·007*	0·000*	0·000*	0·000*	0·005*	0·000*	0·000*	0·000*	0·008*	0·002*
Carbon balance (recovered/supplied)	5·33/6·00	3·33/6·53	4·45/6·00	3·90/6·46	5·27/6·00	5·70/6·74	5·61/6·00	4·69/6·00	5·66/6·00	5·94/6·09	6·62/6·00	4·25/6·00
Redox balance (−/+)	—	1·02/1·09	—	0·67/0·92	—	1·08/1·52	—	1·56/1·32	—	0·81/0·81	—	0·63/0·63
Acid balance (estimated/bicarbonate)	0·94/1·40	1·47/2·76	0·90/1·17	1·95/2·79	0·98/1·19	2·54/2·50	0·78/1·01	1·18/1·08	2·02/2·22	2·00/2·34	1·91/2·53	0·77/1·61

	T. rhodesiense		*T. gambiense*		*T. brucei*		*T. evansi*		*T. equinum*		*T. equiperdum*	
	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2	CO_2/air	CO_2/N_2
Duration of experiment (min.)	60	60	75	75	25	35	30	30	30	30	37	37
Dry wt./vol. (mg./ml.)	14·6/4·0	14·6/4·0	18·2/4·0	18·2/4·0	24·6/4·0	27·7/4·5	14·9/4·0	14·9/4·0	16·9/4·0	16·9/4·0	21·7/4·0	21·7/4·0
Glucose used (μmoles)	21·82	24·40	18·15	30·75	38·15	22·50	29·90	23·05	27·22	23·85	22·85	27·45
R.Q.	0·11	—	0·03	—	0·09	—	0·09	—	0·09	—	0·10	—
Acid (bicarbonate)	1·78	1·19	2·20	1·15	1·87	**1·33**	1·85	1·15	1·83	1·04	1·72	1·11
O_2	1·18	—	1·07	—	0·65	—	0·67	—	0·66	—	0·59	—
CO_2	0·13	0·05	0·03	0·00	0·06	0·03	0·06	0·04	0·06	0·03	0·06	0·03
Glycerol	**0·38**	**1·03**	0·00	**0·92**	**0·58**	**1·13**	**0·47**	**1·00**	**0·28**	**1·01**	**0·60**	**1·10**
Ethanol	0·00	0·00	—	—	0·00	0·00	0·00*	0·00*	0·00	0·00	0·00	0·00
Lactic acid	0·01	0·05	0·00	0·02	0·00	0·04	0·05	0·08	0·02	0·07	0·02	0·03
Pyruvic acid	**1·56**	**0·83**	**1·72**	**0·84**	**1·43**	**0·87**	**1·37**	**0·85**	**1·50**	**0·77**	**1·30**	**0·88**
Acetic acid	0·00*	0·00*	0·03*	—	0·00*	0·00*	0·01	0·07	0·07*	0·10*	0·03	0·03
Succinic acid	0·02	0·04	0·00	0·00	0·00	0·00	0·00*	0·00*	0·00	0·00	0·06	0·03
Citric acid	0·025*	0·010*	—	—	—	—	—	—	—	—	—	—
Carbon balance (recovered/supplied)	6·21/6·00	6·00/6·00	5·25/6·00	5·34/6·00	6·09/6·00	6·15/6·00	5·75/6·00	5·93/6·00	5·60/6·00	5·78/6·00	6·12/6·00	6·24/6·00
Redox balance (−/+)	—	1·00/1·03	—	0·84/0·92	—	0·93/1·13	—	0·93/1·00	—	0·83/1·01	—	0·97/1·10
Acid balance (estimated/bicarbonate)	1·61/1·78	0·96/1·19	1·75/2·20	0·86/1·15	1·43/1·87	0·91/1·33	1·43/1·85	1·00/1·15	1·59/1·88	0·94/1·04	1·47/1·72	1·00/1·10

* Metabolite determined in a separate but similar experiment.

Baernstein (1953) reports bands at 532, 556 and 604 mμ. No trace of a cytochrome *c* band could be detected, even on examination by using liquid nitrogen.

Spectrosopic examination of preparations of *T. rhodesiense*, *T. equiperdum* or *T. congolense* failed to reveal any cytochrome bands; occasional shadows observed in the spectra were probably due to traces of blood cells which are difficult to remove completely, even with extensive washing. Such results do not mean that no cytochrome pigments are present in these three species, but indicate that, if present, they can occur only in traces.

Table 2. *Effect of cyanide and iodoacetate on the respiration of six species of trypanosomes*

Trypanosomes in Warburg manometers in vol. of 1·2 ml. of Ringer-phosphate–glucose or heparinized whole blood; gas phase air; temp. 37° unless otherwise indicated. In experiments with cyanide, balanced KOH–KCN mixtures used in centre wells as recommended by Robbie (1946). Inhibitor (0·3 ml.) added from side bulb and respiratory inhibition estimated over period of usually 1 hr. Results are expressed as percentage inhibition of normal respiration and are the average of three to five determinations. Data for *S. oncopelti* from Ryley (1955).

	Iodoacetate					Cyanide			
Concn. (M) ...	$3{\cdot}3\times10^{-3}$	10^{-3}	$3{\cdot}3\times10^{-4}$	10^{-4}	$3{\cdot}3\times10^{-5}$	$0{\cdot}46\times10^{-3}$	10^{-4}	$0{\cdot}46\times10^{-4}$	10^{-5}
				(*a*) Ringer-phosphate–glucose					
T. cruzi (culture; 30°)	45	30	22	19	2	85	80	71	52
S. oncopelti (30°)	—	55	31	8	—	88	65	37	—
T. lewisi	93	90	83	58	—	96	92	81	67
T. congolense	72	67	62	38	7	10	11	10	9
T. vivax	75	77	63	57	29	5	−7	−3	−1
T. rhodesiense	76	73	67	33	0	−14	−4	4	−9
				(*b*) Whole blood					
T. cruzi	32	16	2	0	0	70	62	64	56
T. lewisi	18	10	2	10	2	74	64	69	62
T. congolense	63	62	50	18	1	20	4	3	2
T. vivax	76	50	—	39	10	9	2	6	7
T. rhodesiense	84	65	61	52	26	3	2	4	1

Table 3. *Cytochrome and succinic oxidase activity in six species of trypanosome*

Trypanosome preparations well washed in Ringer-phosphate solution, suspended in water and shaken with grade 11 Ballotini glass beads in a disintegrator (Mickle, 1948) at room temperature. Strong phosphate buffer (pH 7·3) added to give a final concentration of 0·025M. Dry wt. of homogenate and final volume of system in manometer as indicated; *p*-phenylenediamine (PPD) (13 mg./cup), succinate (0·03M final) and cytochrome *c* ($6{\cdot}5\times10^{-5}$M final) added from side bulb. Figures give oxygen uptake (μl.) observed over the first hour of incubation, and, in brackets, the corresponding Q_{O_2}; figures for systems containing *p*-phenylenediamine + cytochrome *c* have been corrected for O_2 uptake observed in the absence of homogenate.

		Oxygen uptake (μl.)					
Trypanosome	Dry wt./ml.	Blank	Blank + cytochrome *c*	PPD	PPD + cytochrome *c*	Succinate	Succinate + cytochrome *c*
T. cruzi (culture; 30°)	33·8/2·1	0 (0)	0 (0)	46 (1·36)	41 (1·21)	24 (0·71)	30 (0·89)
T. lewisi	44·0/3·1	—	—	91 (2·07)	225 (5·12)	—	—
	6·2/2·3	7 (1·13)	—	—	—	65 (10·48)	128 (20·65)
S. oncopelti (30°)	52·8/2·5	20 (0·38)	—	377 (7·15)	356 (6·75)	158 (3·00)	207 (3·92)
T. congolense	34·8/2·1	56 (1·61)	58 (1·66)	89 (2·56)	236 (6·79)	73 (2·10)	103 (2·96)
T. vivax	41·2/2·1	22 (0·53)	18 (0·44)	24 (0·58)	42 (1·02)	113 (2·74)	132 (3·20)
T. rhodesiense	31·7/2·1	53 (1·67)	58 (1·83)	84 (2·65)	160 (5·05)	60 (1·89)	78 (2·46)

Oxidase systems

The ability of homogenates to oxidize *p*-phenylenediamine and succinate, and the effect of added cytochrome *c* on any such oxidation, have been determined; results from typical experiments are given in Table 3. With *T. cruzi*, *T. lewisi* and *S. oncopelti*, oxygen uptake in such systems continues at a constant rate for at least 2 hr. With the other three species, the rate of oxygen uptake gradually decreases, being about one-third the initial rate after 2 hr. (see Figs. 1 and 2); the respiration of such homogenates is more stable than that of intact cells, where respiration has usually completely ceased by this time.

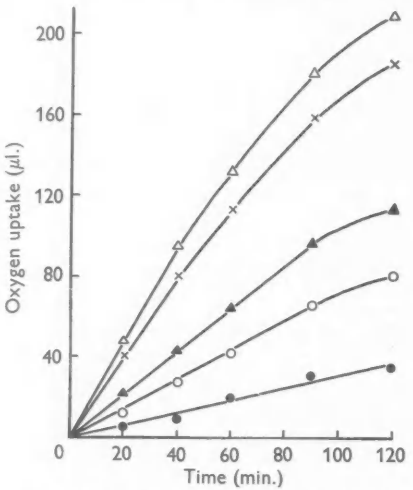

Fig. 1. Oxidase activity in *T. vivax* homogenate. System as described in Table 3. ●, Homogenate with or without cytochrome *c*; ○, homogenate + *p*-phenylenediamine; ▲, homogenate + *p*-phenylenediamine + cytochrome *c*; ×, homogenate + succinate; △, homogenate + succinate + cytochrome *c*.

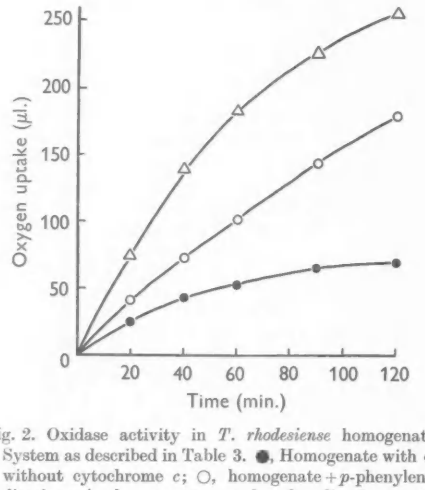

Fig. 2. Oxidase activity in *T. rhodesiense* homogenate. System as described in Table 3. ●, Homogenate with or without cytochrome *c*; ○, homogenate + *p*-phenylenediamine; △, homogenate + *p*-phenylenediamine + cytochrome *c*.

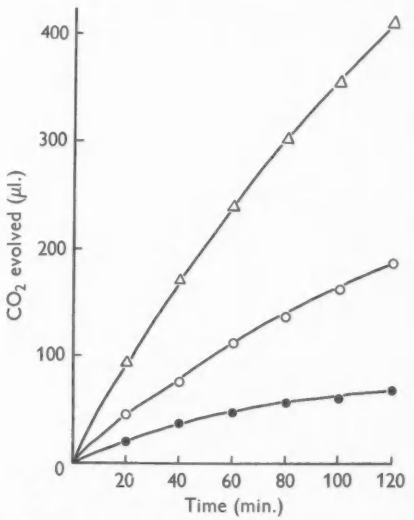

Fig. 3. Dehydrogenase activity in *T. cruzi* homogenate. Each manometer cup contained 0·02 M-$NaHCO_3$, 0·2 ml. of 11% (w/v) $K_3Fe(CN)_6$, 0·08 M succinate or lactate and homogenate (24·6 mg. dry wt.) in total vol. of 2·5 ml.; gas phase $CO_2 + N_2$ (5:95). Temp. 30°. ●, Blank; ○, lactate; △, succinate.

Although the succinic-oxidase activity of homogenates of *T. cruzi* is very low, and the oxygen uptake in the presence of succinate is not stimulated by $3{\cdot}2 \times 10^{-6}$ M methylene blue, such homogenates show high succinic-dehydrogenase activity as determined by the method of Quastel & Wheatley (1938); this is illustrated by Fig. 3, which also indicates the presence of lactic dehydrogenase.

DISCUSSION

The carbohydrate metabolism of all trypanosomes so far studied is characterized by incomplete oxidation of the substrate; this is most marked with the

brucei–evansi group. Thus Reiner, Smythe & Pedlow (1936) found that *T. equiperdum* converted glucose into pyruvic acid under aerobic conditions, or into a mixture of pyruvic acid and glycerol under anaerobic conditions, and Harvey (1949) obtained similar results with *T. hippicum*. Marshall (1948), using *T. evansi*, and Brand, Tobie, Mehlman & Weinbach (1953), using *T. gambiense*, found that pyruvic acid accounted for most of the glucose degraded under aerobic conditions. Fulton & Stevens (1945) claimed that *T. rhodesiense* produced lactic, formic, acetic and succinic acids and ethanol, besides pyruvic acid and glycerol; their data, however, do not provide a ready evaluation of the amounts of these metabolites produced in terms of glucose utilized. Glowazky (1937), using spot tests, found that lactic and oxalic acids, as well as pyruvic acid, were produced by *T. brucei*. The present results indicate that, in the six species of this group studied, pyruvic acid and glycerol account for almost all the glucose disappearing, and the other metabolites can be produced in only insignificant quantities; the R.Q. in all these cases is very small, but it does seem that a little carbon dioxide is produced. With *T. rhodesiense*, no acetic acid or ethanol, and only traces of lactic and succinic acids, could be detected.

In contrast to this, *T. cruzi*, *T. lewisi*, *T. congolense*, *T. vivax* and *S. oncopelti* degrade glucose beyond the pyruvic acid stage, and produce appreciable quantities of carbon dioxide. *T. cruzi* and *T. lewisi* convert about half the glucose molecule into carbon dioxide, the rest appearing as succinic, lactic and acetic acids; glycerol, ethanol and formic acid production in these species is negligible. The detection of small amounts of citric acid in metabolic solutions of *T. cruzi*, *T. vivax*, *T. rhodesiense* and *S. oncopelti* suggests that the tricarboxylic acid cycle is operating in these organisms, and that traces of this key metabolite leak out of the cell; with *T. rhodesiense*, however, the cycle cannot be very important, as the total yield of carbon dioxide is very small. *T. congolense* is similar to *T. lewisi* and *T. cruzi*, except that smaller amounts of carbon dioxide and larger amounts of acid are produced.

In a recent study of the metabolism of *T. congolense*, Agosin & Brand (1954) found that succinic acid was produced only in traces; their experiments, however, were carried out in the absence of carbon dioxide. In the case of *S. oncopelti* it has been shown (Ryley, 1955) that the amount of succinic acid formed depends on the carbon dioxide tension, and this probably explains the different results obtained with *T. congolense*. Agosin & Brand also found an oxygen/glucose ratio of 2·40, their determinations being carried out in whole-rat blood or rabbit serum. We obtained the much lower value of 0·50, using trypanosomes in Ringer-bicarbonate medium, but in plasma a value of 2·11 was obtained; with *T. lewisi* a ratio of 3·59 was found in plasma, as compared with 3·17 in an inorganic medium. Possibly, in the more fragile trypanosomes metabolizing in inorganic media, aerobic processes decay before anaerobic metabolism, so that the oxygen/glucose ratio decreases.

It is interesting to compare the different hydrogen-accepting systems coupled with anaerobic oxidations. In the *T. rhodesiense* group, oxidation–reduction results in the production of glycerol, while with *T. cruzi* and *T. lewisi* no glycerol is formed, but instead two alternative pathways lead to the production of lactic and succinic acids. *T. congolense* and *T. vivax* are intermediate between these groups, producing glycerol and succinic or lactic acids. *S. oncopelti* is unique among the protozoa in that it is able to form ethanol.

From the cyanide-inhibition experiments reported, it can be seen that *T. rhodesiense* and *T. vivax* are insensitive to the inhibitor, *T. congolense* shows a slight sensitivity, and the other three species are very sensitive to cyanide; cytochrome pigments have been detected only in these last three trypanosomes. The cytochrome system of *T. cruzi* is unusual in that cytochrome *c* seems to be absent. Baernstein & Tobie (1951) first noticed this, and reported that respiration, although sensitive to cyanide, was not affected by carbon monoxide.

Homogenates of *T. lewisi* and *S. oncopelti* have been found to show succinic-oxidase and cytochrome-oxidase activity, which can be stimulated by added cytochrome *c*. In *T. cruzi* oxidase activity in the experimental systems used is very low, and is not affected by exogenous cytochrome *c*. The results obtained with *T. vivax* and *T. rhodesiense* are very puzzling; respiration is not affected by cyanide, succinate does not seem to be involved in their metabolism, and *T. rhodesiense* at least contains no detectable cytochrome pigments. In the experimental systems used, however, activity is marked, particularly the succinic oxidase of *T. vivax* and the cytochrome oxidase of *T. rhodesiense*.

One of the most interesting problems in the trypanosome field concerns the difference between the blood stream and the invertebrate or cultural forms of the parasite. For *T. cruzi*, the results presented here do not reveal any marked difference in the two forms. With *T. gambiense*, Brand & Johnson (1947) found the cultural form was slightly sensitive to cyanide, but the blood-stream form was not (Brand & Tobie, 1948). Although the blood-stream form of this parasite degrades glucose only as far as the pyruvic acid stage, Brand, Weinbach & Tobie (1954) found that the cultural

form, which corresponds to the stage found in the insect vector, respired with an R.Q. of unity and produced large amounts of succinic and acetic acids, as well as smaller amounts of other metabolites; it seems that adaptation to life in the animal has resulted in the loss of much catabolic activity.

SUMMARY

1. *Trypanosoma rhodesiense*, *T. gambiense*, *T. brucei*, *T. evansi*, *T. equinum* and *T. equiperdum* degrade glucose to a mixture of pyruvic acid and glycerol; glycerol production is favoured by anaerobic conditions. The R.Q. is small, usually less than 0·10.

2. *T. cruzi* and *T. lewisi* convert glucose into a mixture of lactic, acetic and succinic acids; *T. congolense* and *T. vivax* produce glycerol, acetic acid and succinic or lactic and pyruvic acids; and *Stringomonas oncopelti* forms glycerol, ethanol, pyruvic and succinic acids. In all cases the R.Q. is high, about unity.

3. The respiration of *T. cruzi*, *T. lewisi* and *S. oncopelti* is very sensitive to cyanide, that of *T. congolense* is slightly sensitive, and the respiration of *T. vivax* and *T. rhodesiense* is insensitive to the inhibitor; cytochrome pigments have been detected in *T. cruzi*, *T. lewisi* and *S. oncopelti*, but not in *T. congolense*, *T. rhodesiense* or *T. equiperdum*.

4. Homogenates of *T. cruzi*, *T. lewisi*, *S. oncopelti*, *T. congolense*, *T. vivax* and *T. rhodesiense* have been examined for cytochrome and succinic-oxidase activity; such activity has been found in all preparations to a varying extent.

It is a pleasure to acknowledge the invaluable technical assistance of Miss Margaret Mitchell.

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The Component Fatty Acids of *Penicillium lilacinum* Fat

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During study in this Laboratory of the fat-forming potentialities of a selection of mould fungi it was found by one of us (S.S.) that, under certain cultural conditions, *Penicillium lilacinum* Thom can give a high yield of a fat which is solid at ordinary temperature and which is of a very low free acidity—characteristics that are very desirable in edible fats. It was considered of interest to investigate the component fatty acids and glycerides of this fat.

EXPERIMENTAL

The fat was obtained by cultivating *P. lilacinum* Thom as surface cultures for 14 days at 25° on the following medium (g./100 ml.): K_2SO_4, 0·011; $NaH_2PO_4,2H_2O$, 0·730; $MgSO_4,7H_2O$, 0·500; $ZnSO_4.7H_2O$, 0·005; $FeCl_3,6H_2O$, 0·016; sucrose, 17; $NaNO_3$, 0·640; made up with distilled water and the pH adjusted to 6·8. The felts were removed and treated as described previously (Singh, Walker & Meara, 1955) for the extraction of the fat. The product thus obtained was solid at room temperature and had a slight

yellowish tinge. On examination according to British Standard Methods of Analysis of Oils and Fats (British Standards Institution, 1950) the fat showed the following characteristics: saponification equiv. 286·9; iodine value 63·7; free fatty acids (as oleic) 0·2%; unsaponifiable matter 2·9%.

Spectroscopic examination of the mixed fatty acids by the method of Hilditch, Patel & Riley (1951) gave $E^{1\%}_{1\,cm.}$ at 268 mμ. (170°, 15 min.), 2; $E^{1\%}_{1\,cm.}$ at 234 mμ. (180°, 15 min.), 119·5. Assuming that the unsaturated acids present are solely of the C_{18} series, the following composition was deduced: triethenoid acids, trace; diethenoid acids, 13·2; monoethenoid acids, 47·6; saturated acids, 39·2%.

Determination of component fatty acids

The mixed fatty acids were separated into three fractions (*A*, *B*, and *C*) by crystallization from acetone at −65°, and subsequent crystallization of the acetone-insoluble portion from ether at −30°. The results are given in Table 1. Each fraction was methylated and fractionally distilled. The saponification equivalents and iodine values of the ester fractions obtained for each group are recorded in Table 2. From the analytical data recorded in Tables 1 and 2 the composition of each acid group and finally of the whole fat was deduced (Table 3) by the method adopted previously (Singh *et al.* 1955).

Table 1. *Fractionation of mixed fatty acids by low-temperature crystallization*

Group	Conditions	Acids: Wt. (g.)	Acids: % (w/w)	Acids: Iodine value	Acids: $E^{1\%}_{1\,cm.}$ (234 mμ.)*
A	Insoluble in 10 vol. acetone at −65° and in 10 vol. ether at −30°	53·80	44·2	8·3	18·7
B	Insoluble in 10 vol. acetone at −65°, soluble in 10 vol. ether at −30°	54·03	44·4	94·9	95·6
C	Soluble in 10 vol. acetone at −65°	13·81	11·4	157·2	676·6

* After isomerization with alkali.

Table 2. *Fractionation of methyl esters of acids of groups A, B and C*

Fraction	Wt. (g.)	Saponification equivalent	Iodine value
	Methyl esters of acids *A*		
A_1	1·42	268·1	0·0
A_2	2·15	271·5	3·2
A_3	2·81	272·1	5·1
A_4	3·15	272·5	5·2
A_5	3·80	272·6	5·7
A_6	3·72	274·7	6·1
A_7	4·30	277·6	7·7
A_8	4·16	279·9	8·8
A_9	3·69	286·1	12·4
A_{10}	2·07	293·2	12·6
A_{11}	1·73	314·1*	22·2
	Methyl esters of acids *B*		
B_1	1·61	286·1	81·2
B_2	1·86	287·2	84·1
B_3	3·50	289·1	84·3
B_4	3·13	289·4	87·7
B_5	3·47	290·4	89·6
B_6	3·83	293·4	91·5
B_7	4·42	295·0	93·1
B_8	3·62	296·4	95·3
B_9	2·51	296·5	95·7
B_{10}	1·84	307·9*	84·2
	Methyl esters of acids *C*		
C_1	1·44	284·7	137·7
C_2	1·39	291·2	150·1
C_3	1·81	293·2	151·0
C_4	2·11	294·8	151·3
C_5	1·52	294·4	152·2
C_6	1·70	312·3*	140·9

* Equivalents of esters (freed from unsaponifiable matter): A_{11}, 313·3; B_{10}, 307·5; C_6, 301·8.

Identification of fatty acids

Palmitic acid (m.p. 61·5–62·8°) and stearic acid (m.p. 69·2–70·0°) were recovered from appropriate fractions, and the identity of the oleic acid was confirmed by its conversion into 9:10-dihydroxystearic acid (m.p. 129–130°). Linoleic acid was characterized by conversion into tetrabromostearic acid (m.p. 112·5–113·2°). None of these samples showed depression of m.p. on admixture with an authentic specimen of the appropriate reference substance.

DISCUSSION

From the figures for component fatty acids of *P. lilacinum* fat recorded in Table 3 it is clear that the proportion of saturated acids in this fat is markedly greater than has been found in most of the fats of microbiological origin which have so far been examined. The palmitic acid constitutes about 75% of the total saturated acids and it accords with the amount usually associated with the fats from cryptogams.

The hexadecenoic acid content of *P. lilacinum* fat is of the order that is normal for this acid in fat-rich micro-organisms. The proportion of hexadecenoic acid in the fat-poor micro-organisms is usually high, but it appears to fall with an increase in the reserve fat (Holmberg, 1948).

The amount of linoleic acid in *P. lilacinum* fat (13·4%) is quite moderate. It appears therefore that the fat should possess inherently good resistance to oxidative rancidity.

The most interesting feature of *P. lilacinum* fat is its close resemblance in component fatty acid

Table 3. *Component fatty acids of* Penicillium lilacinum *fat*

	Acid groups (%)*			Total fatty acids excluding unsaponifiable matter (%)	
	A (44·2%)†	*B* (44·4%)†	*C* (11·4%)†	(w/w)	(mol.)
Myristic	0·3	—	—	0·1	0·1
Palmitic	67·6	5·3	—	32·3	34·4
Stearic	21·0	0·3	—	9·4	9·0
Arachidic	3·2	—	—	1·4	1·2
Hexadecenoic	—	6·8	3·5	3·4	3·7
Oleic	5·5	75·4	23·1	38·6	37·3
Linoleic	2·4	9·6	70·6	13·4	13·1
Unsaturated C_{20}	—	2·6	2·4	1·4	1·2
Unsaponifiable	—	—	0·4	—	—

* Component acids as percentage (w/w) of group. † Groups as percentage (w/w) of total acids.

Table 4. *Component acids of pig fat, palm oil, and* Penicillium lilacinum *fat*

	Iodine value	Saturated				Unsaturated				
		C_{14}	C_{16}	C_{18}	C_{20}	C_{14} (−2·0 H)	C_{16} (−2·0 H)	C_{18} (−2·0 H)	C_{18} (−4·0 H)	C_{20-22} (−2·0 H)
Pig*	60·0	1·3	28·3	11·9	—	0·2	2·7	47·5	6·0	2·1
Palm†	60·0	1·6	32·3	5·5	—	—	—	52·4	8·2	—
P. lilacinum‡	63·7	0·1	32·3	9·4	1·4	—	3·4	38·6	13·4	1·4

* Hilditch, Lea & Pedelty (1939). † Bank, Dean & Hilditch (1935). ‡ Present investigation.

composition to pig fat and palm oil (Table 4). In the triglyceride molecules of the latter fats the fatty acids are not arranged according to the general rule of even distribution, and these fats contain a higher proportion of fully saturated glycerides. It will be of interest to ascertain the glyceride structure of *P. lilacinum* fat. This is now under investigation and will be reported in a later communication.

SUMMARY

1. The fat produced by *Penicillium lilacinum* grown in surface culture on a chemically defined medium of sucrose and inorganic salts is solid at room temperature and has a very low free acidity (0·2% as oleic acid).

2. The component fatty acids of this fat are: myristic, 0·1; palmitic, 32·3; stearic, 9·4; arachidic, 1·4; hexadecenoic, 3·4; oleic, 38·6; linoleic 13·4; C_{20} unsaturated acids, 1·4%.

3. The hexadecenoic acid content, like that of fats from other fat-rich micro-organisms, is low.

4. The moderate content of linoleic acid in this fat implies low liability to oxidative rancidity.

The work forms part of an investigation undertaken at the suggestion of the Committee on Chemical Microbiology of the Medical Research Council, and one of us (J.S.) desires to thank the Committee for a personal grant. The authors are indebted to Dr A. R. Thompson of this College for spectroscopic determinations.

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Cholesterol Metabolism

7. CHOLESTEROL ABSORPTION AND EXCRETION IN MAN*

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The experiments to be described were designed primarily to determine the extent of absorption in man of crystalline cholesterol and of cholesterol taken in the form of eggs. The constituents of the ether–ethanol extracts of the faeces before and after the ingestion of the cholesterol were examined to determine the nature of the excretory products.

A preliminary account of the findings has been given by Cook & Edwards (1951), Edwards & Cook (1951) and Cook, Edwards & Riddell (1952).

DIET AND METHODS

Diet. Two separate experiments on the same male subject (age 45 years, wt. 71 kg.) were carried out, a period of 10 months elapsing between each experiment. Apart from the special egg diet which contained twenty eggs, no alterations were made in dietary habit. The subject's health and appetite were uniformly good and his weight was constant.

The first experiment consisted in the collection of faeces for 5 days on a normal diet and on the 3 days following the ingestion of 10 g. of cholesterol (Glaxo Recrystallized) suspended in 50 g. of olive oil. The cholesterol was taken in two lots of 5 g. in oil, one at 1.30 p.m. and the other at 6 p.m. In the second experiment twenty eggs made into various dishes were consumed over a period of 15 hr. Faeces from the normal diet were collected 2 days before the ingestion of the egg diet and for the 4 days following. No urine was collected, as loss of sterol by this route is negligible (see Cook, 1952).

Collection and treatment of faeces. During the normal period and after ingestion of crystalline cholesterol bowel habit was regular, but about 3 hr. after the first meal on the egg diet there was a transient loosening, the material collected being designated 'transition'. Thereafter regular habits were maintained.

The faeces were collected daily, normally after breakfast, into a tared cellophan bag and weighed. The bag and its contents were then dried *in vacuo* in a steam-jacketed vessel to constant weight and extracted in a continuous extractor with ether until no colour was visible in the extract. The material was then removed from the extractor, powdered in a mortar and the extraction was completed with ethanol. The solvents were removed and the extracts were weighed. In the first experiment the ether-soluble and the ethanol-soluble extracts were weighed and analysed separately, but in the second experiment the two extracts were combined. The reported results are for the combined extracts.

* Part 6: Cook, Edwards, Riddell & Thomson (1955).

Analysis and treatment of extracts. Samples were analysed for free, total and saturated sterols by the methods previously described (Riddell & Cook, 1955). The procedures used for saponification, removal of unsaponifiable matter (UM), and recovery of steam-volatile and ether-extractable acids and for the fractionation of these were the same as those described by Riddell & Cook (1955) and Edwards & Cook (1955).

Eggs. Four eggs were dried with Na_2SO_4 on a water bath. The mixture was powdered and extracted and the lipid was fractionated as for the faeces.

Blood. This was collected in oxalated tubes and the plasma separated. Total ether-soluble lipid, free and total sterol were determined on certain samples.

Chromatography. Adsorption chromatography on alumina was used for fractionating the unsaponifiable matter and the methyl esters of the acids by the methods described previously (Riddell & Cook, 1955; Edwards & Cook, 1955).

RESULTS

The average composition of the normal diet and the composition of the egg diet are given in Table 1, the food values being calculated from the tables of

Table 1. *The main constituents of the diets used*

The values given are g./day

	Average normal diet	Egg diet
Carbohydrate	303	412
Fat	134	357
Protein	85	175
Cholesterol	0·58	6·9
Phytosterols	0·08	0·15
Total calories per day	3140	6120

McCance & Widdowson (1946). The values for cholesterol in ordinary foods were taken from those determined by Okey (1945) and the values for plant sterols from those collected by Lange (1950). The crystalline cholesterol preparation contained 98% of cholesterol (Riddell & Cook, 1955). The following values per egg were found: total extract 7·1 g., UM 412 mg., total sterol 343 mg. Twenty eggs therefore contain 8·25 g. of UM and 6·9 g. of sterol. The total digitonin-precipitable sterol contained 4% of 'fast acting' sterols, mainly lathosterol (Fieser, 1951). The diet contained about

Table 2. *Data on faecal excretion on normal and cholesterol-containing diets*

Particulars of the diets are given in Table 1 and the text. In Expt. 1 faeces were collected for 5 days, whilst the subject was on a normal diet (days 1–5). On the 5th day the subject ingested 10 g. of crystalline cholesterol in two divided doses. Faeces were collected for 3 days after cholesterol feeding. In Expt. 2 the preliminary period on a normal diet was 2·5 days; then twenty eggs were consumed over 15 hr. and the faeces were collected over the following 4 days. The figures for the period 1–5 days in Expt. 1 are average values in the case of fresh wt. and dry wt. of faeces, whilst the other values shown for this period were obtained by dividing the total figures for a 5-day collection by 5. Numbers in brackets denote range of values, UM is unsaponifiable matter and — denotes not determined. All figures are given in g.

	Days of expt.	Faeces		Wt. of ether–ethanol extract	Wt. of UM	Sterol			Acids	
		Fresh wt.	Dry wt.			Total	Free	Saturated	Ether-extractable	Steam-volatile
Expt. 1										
Normal diet	1–5	140 (98–192)	34 (22–44)	8·5	1·5	0·4	0·35	0·4	2·6	0·5
After cholesterol ingestion	1	157	35	15·4	6·1	5·6	5·3	4·1	2·9	2·2
	2	119	24	7·4	2·9	1·9	1·6	1·1	1·5	0·7
	3	107	29	9·0	3·4	2·4	1·9	1·3	1·9	0·9
Expt. 2										
Normal diet	1	101	20	4·1	0·7	0·4	0·3		0·9	—
	2	141	28	7·8	1·4	0·3	0·2	1·1	1·7	—
	2·5	154	32	6·2	1·1	0·4	0·1		1·3	—
Average for days 1–2·5		158	32	7·2	1·3	0·5	0·3	0·4	1·6	—
After ingestion of 20 eggs	1	143	27	8·9	3·4	1·8	1·5	1·4	2·2	—
	2	153	30	9·8	1·8	1·2	0·8	0·5	2·9	—
	3	186	37	11·2	2·0	0·8	0·7		3·3	—
	4	161	36	11·5	1·7	0·5	0·3	0·4	3·9	—

10 g. of unavailable carbohydrate (fibre) per day and the consumption of alcohol yielded about 10% of the total calories.

The values in Table 2 for the normal excretion have been calculated on an average daily basis by dividing the totals in the normal period of the first experiment by 5; in the normal period of the second experiment the transition excretion has been counted as a half day and the total values have been divided by 2·5. On such a daily basis the values for weight of faeces, extract, UM and sterol excreted for the two periods are very similar. The amount of ether-extractable acids is less in the normal period of the second experiment.

On the first day after ingestion of crystalline cholesterol there was a marked increase in the amount of extract, UM and total sterol. The excretion of UM and of sterol continued for the next 2 days. There was an increase in the excretion of ether-extractable acids on the first day after ingestion but the value thereafter fell to lower than normal. There was a marked rise in the amount of steam-volatile acids on the first day, but thereafter the amount decreased.

On the egg diet the amount of extract increased for the 4 days after ingestion. The excretion of UM was increased as was that of the total sterol. There was a marked increase in the excretion of ether-extractable acids. Further consideration of the excreted material is given later.

Absorption of cholesterol

From the values given in Table 2 the apparent absorption of cholesterol may be calculated. In Table 3 are given the values for the absorption of unsaponifiable matter and of sterol. The small additional amounts of UM and of sterol ordinarily ingested are not shown. Calculated on the basis of UM the absorption for crystalline cholesterol is 21% and for egg 55%. On a sterol basis the absorptions are 11 and 67% respectively. There is thus a marked difference in absorption. The general conclusion may be drawn that, in one subject, of 10 g. of crystalline cholesterol ingested, about 15% was absorbed over 3 days (or about 8 mg./kg. body wt./day), and of 6·9 g. of egg cholesterol ingested about 60% was absorbed over 4 days (or about 15 mg./kg. body wt./day).

Level of sterol in plasma. The values for free and total cholesterol were determined before and 18 hr. after the ingestion of crystalline cholesterol but there was no demonstrable increase. With the egg diet the sample of plasma obtained 6 hr. after the beginning of the diet, when about 4 g. of cholesterol and 210 g. of fat had been consumed, showed a significant rise in total lipid and in total cholesterol and the percentage of ester had risen above the

Table 3. *Calculated values for sterol absorption after ingestion of crystalline cholesterol (Expt. 1) and after ingestion of twenty eggs (Expt. 2)*

Experimental details and data on which the calculations are based are given in Table 2. UM denotes unsaponifiable matter. All values other than those in the last horizontal row are in g.

	Expt. 1		Expt. 2	
	UM	Sterol	UM	Sterol
Amount eaten additional to that present in normal diet (*A*)	10·0	9·8	8·3	6·9
Total amount excreted after cholesterol feeding* (*B*)	12·4	9·9	8·9	4·3
Normal excretion* (*C*)	4·5	1·2	5·2	2·0
Excess excreted (*B* − *C*)	7·9	8·7	3·7	2·3
Amount absorbed (*A* − *B* + *C*)	2·1	1·1	4·6	4·6
Amount absorbed (%)	21	11	55	67

* This was over a period of 3 days in Expt. 1 and of 4 days in Expt. 2.

Table 4. *Contents of lipid and of total and free cholesterol in plasma before and at various times after beginning of the egg diet*

For details of diet see Table 1 and text.

	Lipid	Cholesterol	
		Total	Free
	(mg./100 ml.)		
Before ingestion of eggs	750	230	100
After 6 hr.	1200	325	90
After 24 hr.	950	230	90
After 4 days	850	215	85

normal value. After 24 hr. the values had returned to normal and were still normal when tested on the fourth day (see Table 4).

Unsaponifiable matter (UM)

The nature of the UM was investigated by two methods. In the first investigation the UM from the normal diet preceding (*N*) and the UM from day 1 (*C*1), days 2 and 3 combined (*C*2–3) and day 4 (*C*4) following the ingestion of egg cholesterol were studied. The separate samples were initially partitioned between aqueous methanol and light petroleum (Haslewood, 1941); the fractions were then recovered and the light-petroleum-soluble moieties were chromatographed on alumina, to yield five primary fractions of comparable nature. In essentials the method was the same as method I of Riddell & Cook (1955).

The second investigation was devised to study the chemical nature of the total UM, and a pool was made of the remaining material from the normal and crystalline cholesterol diet experiment and material from the egg-diet experiment from which the greater part of the coprostanol and cholesterol had been removed.

Method 1

The yields (as g. and as percentage of total UM), appearances, $[\alpha]_D$, iodine values, and sterol estimations by quantitative Liebermann–Burchard reaction (LB) and by gravimetric digitonin precipitation (G) are given in Table 5. The eluents used to obtain the various fractions are also given.

Aqueous-methanol-soluble. This fraction increased slightly after the ingestion of eggs, reaching 10% of the total UM on the fourth day. The fraction contains the most polar compounds and was shown by paper partition chromatography (see Riddell & Cook, 1955) to contain diols and triols. A more detailed account of the composition is given below.

Fraction 1. This increased slightly in amount after the ingestion of eggs, but the materials were similar in appearance and properties. They were white crystalline waxes (m.p. 38–42°) with no optical activity. The LB test was 'fast' but gave a low sterol value, and no material was precipitable with digitonin. This fraction is considered to consist mainly of saturated and unsaturated hydrocarbons (see below).

Fraction 2. The fractions from *C*1 and *C*2–3 were increased above that of *N*. The acetyl value for the *N* fraction was 150 and for the combined *C* fractions it was 100. The gummy fractions could not be crystallized either before or after acetylation. The fraction is considered to consist mainly of higher alcohols with an admixture of sterols.

Fraction 3. This increased slightly after the ingestion of eggs, the effect being most marked in sample *C*4. The value for digitonin-precipitable sterol was greater than that obtained by the LB test. The *N* and *C* fractions all gave a strongly positive Zimmermann test, indicating the presence of 3-ketosteroids. Spectrophotometric examination indicated the presence of cholest-4-en-3-one in the *C* fractions.

The *N* and all the *C* fractions were combined (0·18 g.), treated with Girard T reagent and the ketonic fraction (0·11 g.) was recovered. Polarographic investigation by the method of Robertson (1955) showed the presence of cholestanone (7·5 mg.) and coprostanone (4 mg.). Chromatography of the 2:4-dinitrophenylhydrazones showed that other constituents were also present.

Fraction 5. The amount of this fraction increased somewhat in *C*1 and was markedly increased in *C*2–3; in *C*4 there was a decrease. The fractions from *N*, *C*1 and *C*1 and *C*2–3 are considered to consist mainly of unsaturated sterols.

Components of fractions 4 *and* 5. The remainders of the fractions of similar nature were combined to form the following pools: fraction 4*N* (1·50 g.); fraction 4*C* (2·84 g.) from *C*1, *C*2–3 and *C*4; fraction 5*N* (0·77 g.); and fraction 5*C* (3·57 g.) from *C*1, *C*2–3 and *C*4. These fractions were chromatographed separately on alumina by the method shown in Fig. 1 and a number of subfractions obtained. The yields and properties of these are given in Table 6.

Fraction 4.1 from both *N* and *C* material was a yellow, low-melting-point, dextrorotatory wax with a low iodine value. No digitonin-precipitable sterol was present and a slight LB reaction was obtained. It is suggested that the fraction contains 3α-stanols. The amount of this fraction is increased after sterol feeding.

Table 5. *Yields and properties of various fractions of unsaponifiable matter* (*UM*) *of lipids obtained from faeces at various periods of the experiments*

The lipid extracts were obtained from faeces while the subject was on a normal diet (*N*) and 1 day (*C*1), 2–3 days (*C*2–3) and 4 days (*C*4) after the ingestion of eggs. The fractions were obtained by chromatography on alumina (Riddell & Cook, 1955). The values for the quantitative Liebermann–Burchard test (LB) and those for the gravimetric digitonin estimation (G) are expressed as percentage of the fraction examined. The proportions of the solvents in eluent mixtures are given as % (v/v). The light petroleum (LP) had b.p. 40–60°.

Fraction and eluents used		*N*	*C*1	*C*2–3	*C*4
Aqueous-methanol-soluble	Wt. (g.)	0·18	0·20	0·14	0·17
	% of UM	5·6	5·9	3·8	10·0
	Appearance		Brown gums		
	$[\alpha]_D$	+2°	+8°	+10°	−8°
(1) LP 100 and LP 80–benzene 20	Wt. (g.)	0·11	0·09	0·06	0·04
	% of UM	3·4	2·8	1·6	2·3
	Appearance		White crystalline waxes		
	$[\alpha]_D$	0	0	0	0
	Iodine value	24	101	20	25
	Sterol LB	5	10	2	15
	Sterol G	0	0	0	0
(2) LP 20–benzene 80 and benzene 100	Wt. (g.)	0·03	0·09	0·01	0·11
	% of UM	0·9	2·8	1·6	2·3
	Appearance		Gold-brown gums		
	$[\alpha]_D$	0	+30°	0	+9°
	Iodine value	97	73	85	58
	Sterol LB	10	2	10	2
	Sterol G	25	10	30	10
(3) Benzene 80–ether 20 and benzene 20–ether 80	Wt. (g.)	0·07	0·08	0·06	0·18
	% of UM	2·2	2·4	1·6	10·6
	Appearance		Yellow-brown gums		
	$[\alpha]_D$	+30°	+25°	+30°	−9°
	Iodine value	40	43	36	56
	Sterol LB	20	60	5	40
	Sterol G	100	80	10	80
(4) Ether 100	Wt. (g.)	1·71	1·70	0·68	0·96
	% of UM	53·2	50·0	18·4	56·5
	Appearance		Yellow crystalline solids		
	$[\alpha]_D$	+20°	+21°	+20°	+16°
	Iodine value	23	11	14	10
	Sterol LB	30	30	20	45
	Sterol G	80	80	90	90
(5) Ether 80–ethanol 20 and graded mixtures up to ethanol 100	Wt. (g.)	0·88	1·07	2·67	0·14
	% of UM	27·5	31·4	72·0	8·2
	Appearance		Brown gums		
	$[\alpha]_D$	−20°	−25°	−30°	+24°
	Iodine value	69	59	61	154
	Sterol LB	50	70	70	70
	Sterol G	80	90	50	90

Fractions 4.2 from *N* and *C* were white crystalline solids of similar melting points and optical rotations. The iodine value of the *C* material was higher than that of the *N* fraction. All the sterol was precipitable with digitonin. The LB test gave a lower value for total sterol. The main component appears to be coprostanol with an admixture of cholesterol (or other Δ^5-stenol) and cholestanol. The fraction is increased after sterol feeding.

Method 2

The remainder of the UM excreted (16·8 g.) before and during the 3 days after the ingestion of crystalline cholesterol and the residual fractions (3·1 g.) from the egg-diet experiment, from which the greater part of the cholesterol and coprostanol had been removed, were combined. The total corresponds to about 10 days' collection.

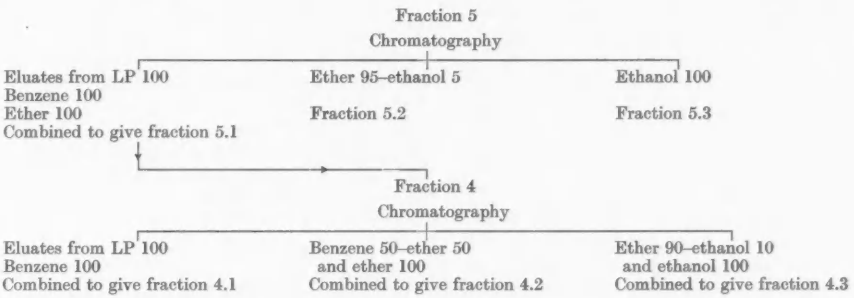

Fig. 1. Separation by chromatography on alumina of the components of fractions 4 and 5 obtained by the chromatography of the unsaponifiable matter from the egg-diet experiment (Table 5). The fractions from *C* 1, *C* 2–3 and *C* 4 were combined. The proportions of solvents in mixtures are given as percentages (v/v). Light petroleum (LP) had b.p. 40–60°.

Table 6. *Yields and properties of fractions obtained from re-chromatography of C fractions 4 and 5 from the egg-diet experiment and of N fractions from normal diet*

For details see text. Sl. = slight positive reaction.

	4.1		4.2		5.2	
	N	*C*	*N*	*C*	*N*	*C*
Wt. (g.)	0·26	1·17	0·93	1·49	0·54	2·60
M.p.	66°	68°	87–89°	88–91°	135–136°	135–136°
$[\alpha]_D$	+14°	+24°	+26°	+29°	−38°	−40°
Iodine values	6	9	5	22	71	57
Sterol (% of fraction):						
Fast-acting	0	0	Sl.	Sl.	1·0	1·4
LB test	Sl.	Sl.	94	86	90	87
Digitonin ppt.	0	0	100	100	100	100

Fraction 5.2 was markedly increased after sterol feeding. The white crystalline solids consist mainly of unsaturated sterols. The properties of the *C* fraction suggest that it was mainly cholesterol mixed with about 10% of cholestanol. The *N* fraction had properties somewhat different from cholesterol and it probably contained phytosterols.

Fraction 5.3 (*N* 24 mg., *C* 95 mg.) and fraction 4.3 (*N* 39 mg., *C* 135 mg.) were gums which gave tests for the presence of diols and triols (Riddell & Cook, 1955). These fractions were not further investigated.

The fractionation procedure was modelled on that of method II of Riddell & Cook (1955), in which the greater part of the cholesterol is removed by crystallization from methanol and the cholesterol-poor unsaponifiable matter (CPUM) fractionated by chromatographic and by chemical methods. The crude cholesterol was crystallized from acetic acid and the materials from the mother liquor were added to the CPUM. There was a working loss of 15%, which may be regarded as having been divided among the various fractions. The results reported are of a preliminary nature and special methods are being developed to separate

the components of the complex fractions. The following fractions were obtained:

Unaltered cholesterol (4·03 g.). This had m.p. 143° and $[\alpha]_D - 39°$. Paper-partition chromatography showed one spot only.

Coprostanol (5·18 g.). This had m.p. 100–101° and $[\alpha]_D + 26°$. Gravimetric and colorimetric estimations indicated that 99 % of this fraction was in fact coprostanol. Paper-partition chromatography showed one spot.

Hydrocarbon fraction (0·13 g.). This was a white crystalline wax, m.p. 38–42°, which showed a bluish fluorescence in ultraviolet light. It had no optical activity. An immediate blue LB test was obtained, but the fraction was not precipitable with digitonin. Analysis of a sample gave: C, 84·7; H, 13·6 %.

Fraction 2 (0·88 g.). This was obtained from the later light-petroleum and the early benzene eluents. The material was a yellow wax which formed an adduct with urea. It had no optical activity and gave an acetyl value of 49. It is suggested that the fraction is a mixture of aliphatic alcohols and other compounds. Cetyl alcohol has been isolated from human faecal UM by Gardner (1921) and by Schoenheimer & Hilgetag (1934), but their yields appear to be small. The nature of the other compounds is unknown but they may be oxidized hydrocarbons of the squalene type and vitamin E-like compounds which are poorly absorbed (Klatskin & Molander, 1952).

Ketones. A yield of 0·56 g. was obtained for the material from the Girard separation, but the yields of cholestanone (16 mg.) and coprostanone (9 mg.) show that a large proportion is unaccounted for. Chromatography of the 2:4-dinitrophenylhydrazones showed that at least five components were present (Robertson, 1955).

Stanols (0·45 g.). This fraction was obtained from the later light petroleum and benzene eluents. It was dextrorotatory, and an analysis gave 20 % of digitonin-precipitable sterol, whereas the LB test gave 7 % of sterol. A yellow colour with a green fluorescence was obtained with concentrated sulphuric acid. It is probable that this fraction contains 3α-stanols in addition to the digitonin-precipitable sterols.

Mixed sterols. A large fraction (4·22 g.) was obtained from the benzene 50–ether 50 eluents. Fast-acting sterols (about 2 %) were present, and digitonin precipitation showed that the fraction consisted mainly of 3β-sterols. Paper-partition chromatography (Riddell & Cook, 1955) indicated a mixture of Δ^5-stenols and of stanols. The fraction contains residual cholesterol and coprostanol, but there is evidence for the presence of other sterols such as phytosterols. (The diet contained a total of about 1 g. of phytosterols during the experimental period.) Windaus & Uibrig (1915) found cholestanol in human faecal UM but gave no yields. Our attempts to isolate this compound were unsuccessful.

Final fraction (1·02 g.). This was obtained from the ether–ethanol eluents, and from the extruded column by boiling with ethanol. The fraction corresponds with the aqueous-methanol-soluble material obtained from the egg-diet experiment. The material was a dark gum with a slight dextrorotation. It contained 2·2 % of nitrogen and gave a marked Lifschütz reaction. Paper-partition chromatography (Riddell & Cook, 1955) showed the presence of cholest-5-ene-3β:7α-diol, cholest-5-ene-3β:7β-diol, cholest-5-ene-3β:24- and/or :25-diol, and cholestane-3β:5α:6β-triol. There was a brown-black spot at the origin after spraying with $SbCl_5$ in $CHCl_3$. The composition of this fraction appears to be similar to the corresponding fraction obtained from rat-faecal UM (Riddell & Cook, 1955).

Acidic component

Acids form a large percentage of the total faecal extracts and the following investigations as to their nature were made.

Steam-volatile acids. These were determined on the faecal extracts of the normal diet preceding, and on the extracts following, the ingestion of crystalline cholesterol. The extracts from the second and third days were combined. The results obtained are shown in Table 7. Expressed on a kg. body wt./day basis there was an increase in the daily excretion of total volatile acids on the first day after the ingestion of crystalline cholesterol, but the amount decreased on the second and third days. The distribution of the fatty acids was of the same general pattern on the normal diet and after the ingestion of crystalline cholesterol.

Table 7. *Steam-volatile acids excreted on the normal diet and after the ingestion of crystalline cholesterol*

	Normal diet (5 days)	Cholesterol diet: Day 1	Cholesterol diet: Days 2 and 3
Total volatile acids (g.)	2·63	2·15	1·57
Acids, as mg./kg. body wt./day	7·4	30·3	11·0
Acids, as percentage of total			
C_2	18	22	17
C_3	18	20	19
C_4	36	39	42
C_5	17	11	13
C_6 and higher	11	9	10

Ether-extractable acids. Two methods were used to study these acids. In the crystalline cholesterol experiment the acids were treated initially with light petroleum and separated into soluble and

insoluble fractions. The soluble fractions were separated by the Twitchell method (see Hilditch, 1949) into 'solid' and 'liquid' acids, which were then investigated. The light-petroleum-insoluble fraction is described below.

In the egg-cholesterol experiment the total acids were converted into the methyl esters and separated by chromatography on alumina. The largest fractions were those obtained from the pure benzene eluates. These fractions were semi-solids and soluble in light petroleum and had no demonstrable optical activity. Other fractions were obtained which were insoluble in light petroleum and are described below. Each fraction from the separate collections was saponified and the recovered acids were separated by the Twitchell method into 'solid' and 'liquid' acid fractions. The acid equivalents and iodine values of these were determined. The results of the separation and the analyses of the light-petroleum-soluble fractions are given in Table 8, where also the total weights of the insoluble fractions are given.

Table 8. *Amounts and properties of fractionated ether-extractable acids in lipids from faeces of a man on normal and on high-cholesterol diets*

For methods of fractionation see text.

		Light-petroleum-soluble fraction							
			Solid acids			Liquid acids			
Diet	Days	Wt. (g.)	% of total soluble acids	Equiv.	Iodine val.	% of total soluble acids	Equiv.	Iodine val.	Total wt. of light-petroleum-insoluble fraction (g.)
Normal	1–5	9·9	58	290	13	42	383	40	3·3
Crystalline cholesterol	1–3	4·6	56	295	12	44	400	53	1·7
Normal	1–2½	2·3	48	266	8	52	285	84	1·6
Egg cholesterol	1	1·5	36	275	11	64	323	68	0·7
	2, 3	4·7	71	257	7	29	311	89	1·5
	4	3·2	70	281	10	30	306	57	0·7

Table 9. *Reversed-phase chromatography of solid fatty acid fractions excreted on normal diets and after ingestion of crystalline cholesterol and of egg cholesterol*

Values are given as percentage of total fraction.

	C_{16} and lower acids	C_{18} and higher acids
Normal diet	60	40
Crystalline cholesterol (days 1–3)	60	40
Normal diet	55	45
Egg cholesterol (day 1)	65	35
(days 2, 3)	62	38
(day 4)	59	41

There was a marked increase in the excretion of light-petroleum-soluble acids after the administration of eggs. There was some increase in the proportion of liquid acids on the first day after ingestion but on subsequent days the proportion of solid acids was greatly increased. No marked changes were observed after the ingestion of crystalline cholesterol.

Solid acids. Some further investigations of the 'solid' acids from the light-petroleum-soluble acid fraction from the normal diet were made. The acids were converted into methyl esters and fractionally distilled *in vacuo*. The following acids (expressed as a percentage of the total) were found: tetradecanoic 12, hexadecanoic (palmitic) 61, octadecanoic 7, C_{20} and higher acids 17, and oleic 4. Palmitic acid is thus the major component.

Reversed-phase chromatography (Howard & Martin, 1950) was carried out on the solid acid fractions, with the results shown in Table 9. The separation of individual acids was not as clear-cut as could be wished, but there was a good separation at C_{16} and C_{18}. The values are given (as a percentage of the total) therefore for C_{16} and lower acids, and for C_{18} and higher acids. In the first experiment the acids excreted after the ingestion of crystalline cholesterol were pooled and there was no difference in the ratios obtained for them and those excreted on the normal diet. With the egg-diet experiment the ratio of C_{16} and lower acids increased after the ingestion of cholesterol, but the normal value was reached after 4 days.

Liquid acids. Apart from the values given in Table 8 no further investigations were made. In the first experiment the acid equivalents are much higher than, and the iodine values less than, those of oleic acid. With the egg-diet experiment the values for the excreted acids on the normal diet are similar to those for oleic acid, but after ingestion of eggs the acid equivalents were increased and in general the iodine values were decreased.

A spectrophotometric investigation gave a value of about 5% for linoleic and linolenic acids in the

liquid acid fraction from the faecal extract on the normal diet. The value was not increased after cholesterol feeding.

It is presumed that the 'liquid' acids are a mixture of C_{18} and higher unsaturated acids, with oleic acid predominating.

Light-petroleum-insoluble acids. The acids excreted on the normal diet (*N*, 3·12 g.) and all those excreted after the ingestion of crystalline cholesterol (*C*, 1·53 g.) were converted into methyl esters and treated with the Girard reagent. The ketonic fractions were dark tars (*N*, 1·09 g.; *C*, 0·79 g.). On treatment with ether a black insoluble portion was obtained which contained 2·5% of nitrogen. The ether-soluble fractions (*N*, 0·84 g.; *C*, 0·46 g.) were dark gums. Polarographic investigation gave waves characteristic of a 3-keto bile acid and a precipitate was obtained with an ethanolic solution of 2:4-dinitrophenylhydrazine. The materials were too dark for polarimetry. They contained about 1% of nitrogen.

The non-ketonic fractions (*N*, 0·86 g.; *C*, 0·45 g.) were gums with no demonstrable optical activity. They were not further investigated.

On the egg diet, the light-petroleum-insoluble fractions were derived from the chromatography of the methyl esters of the acids from the normal feeding period, and from the first, the combined second and third, and the fourth days after ingestion. Fractions 2 were obtained from the 50% benzene–50% ether eluents, fractions 3 from the pure ether eluents and fractions 4 from the 50% ether–50% ethanol eluents. A final fraction was obtained from the extruded column with boiling ethanol containing 3% (v/v) of acetic acid.

The fractions 2 in all cases were light-green semi-solids which were slightly dextrorotatory. Fractions 3 were green tars and were dextrorotatory. Both fractions gave precipitates with ethanolic 2:4-dinitrophenylhydrazine. Fractions 4 were dark-green tars with a nitrogen content of 10%.

The yields and appearances of the fractions from the normal diet and after the ingestion of eggs were similar; there was no increase after the intake of cholesterol.

The light-petroleum-insoluble fraction contains coloured nitrogen-containing acids derived from bile or from ingested chlorophyll, and keto acids which are possibly related to bile acids. The fraction is being studied further.

Pigments

All the neutral fractions were coloured to a greater or less degree, and coloured bands were present on the alumina columns. These were due to carotenoids present in the diet. The daily intake of carotenoids as i.u. of carotene per day was about 5000, which is equivalent to about 30 mg. for 10 days. The carotenoids are known to be poorly absorbed (Hume & Krebs, 1949). Spectrophotometric investigation of one fraction showed the presence of zeaxanthin and xanthophyll.

The acid fraction when converted into the methyl esters showed green bands, presumably of the chlorophylls, and brown-red bands, probably of the porphyrins. A brilliant red band was also seen. The coloured components appeared in the most polar fractions.

DISCUSSION

Products of excretion

After the ingestion of cholesterol there is an increased excretion of UM, which consists largely of sterols. The results given in Table 2 show that after the ingestion of crystalline cholesterol there is a greater excretion of UM which is mainly sterol. The amount of saturated sterol is greatly increased on the first day after cholesterol ingestion, and thereafter the amounts of saturated and unsaturated sterols are nearly equal. With the egg diet, on the first day after ingestion, the saturated sterols form a large part of the sterol fraction, but on later days the amounts of unsaturated sterols increase. The trend of values seems to indicate that on the first day after cholesterol ingestion there is an excretion of unchanged cholesterol and a marked intestinal conversion of cholesterol into coprostanol. The increased sterol excreted on subsequent days may represent re-excretion of absorbed cholesterol via the bile and subsequent intestinal conversion into coprostanol.

The difference between UM and total sterol precipitable by digitonin is due in part to compounds that are not steroids, such as hydrocarbons and aliphatic alcohols, but there is evidence that 3α-stanols (fraction 4.1) are increased in amount. The limited information on the ketones shows that there is no great increase after cholesterol feeding. The small amount of 'fast-acting' sterols present in fraction 5.2 is slightly increased after sterol feeding, as are the most polar compounds, consisting in part at least of the diols.

The neutral fraction of the faecal extract of man, both before and after cholesterol ingestion, is similar to that of the rat except that the conversions of the cholesterol in man seem to be much slower (Cook *et al.* 1955).

The light-petroleum-soluble acids are a mixture of fatty acids and the light-petroleum-insoluble acids are a mixture, one part of which is possibly keto bile acids. From the results given in Table 8 it will be seen that there was an increased excretion of the total ether-soluble acids on the first day after the ingestion of crystalline cholesterol but on sub-

sequent days the amount decreased. On the egg diet there was a continued increase in the excretion of total acids, which was paralleled by an increase in the solid acid fraction (mainly palmitic acid). There was a small rise in the excretion of esterified sterol as measured by the difference between free and total sterol, but this was not enough to account for the large increase in solid acids. It is tempting to relate the appearance of fatty acids to the disappearance of cholesterol, but such evidence is indirect. Unlike the findings with the rat, there was no coincidental increase in the excretion of the light-petroleum-insoluble fraction.

Unfortunately the steam-volatile acids were not determined in the egg-diet experiment, and only general conclusions can be drawn. An interesting feature is the amount of volatile acids present in the normal excretion. The main acids present are acetic and butyric acids. This confirms the earlier findings of Brieger (1877) and Cecchini (1923). Brieger noticed that a small amount of *iso*butyric acid was present in the volatile acid fraction.

served by us. The values for the tritium-labelled cholesterol in the experiments of Biggs *et al.* (1952) were obtained for patients in a poor physical condition and, when calculated on an estimated kg. body wt. basis, show poor absorption. Their experiments and those of Favarger & Metzger show clearly that the labelled cholesterol enters the blood stream. Ungar & Dorfman (1953) have demonstrated absorption of ^{14}C-labelled cholesterol into the blood stream, but only minute amounts were converted into steroid hormones. All workers on this subject are agreed that crystalline cholesterol is poorly absorbed and that the process of excretion is continued over a number of days. This result contrasts markedly with the ready absorption in experimental animals (see Cook & Thomson, 1951).

Our results show that in the same subject egg cholesterol was more readily absorbed. The presence of the large amount of fat, and in particular lecithin, may be responsible for this effect. Egg-yolk fat is readily digestible. Langworthy &

Table 10. *Reported values for the absorption of cholesterol in man, based on balance experiments*

Form of cholesterol eaten	No. of subjects	Wt. (kg.)	Period of study (days)	Dietary sterol (g.)	Sterol excreted (g.)	Sterol absorbed: Amount (g.)	Sterol absorbed: mg./kg. body wt./day	Reference
Crystalline sterol (5·0 g.) in oil (100 g.)	4	—	3	Range 5·8–6·5 Mean 6·0	1·4–4·2 2·7	— 3·3	— —	Bürger & Winterseel (1929)
Crystalline sterol (9·8 g.) in oil (50 g.)	1	71	3	11·5	9·9	1·6	7·5	Present study
Egg sterol (6·9 g.) and fat (357 g.)	1	71	4	9·2	4·3	4·9	17	Present study
Deuterium-labelled (4·0 g.) in fat (25 g.)	1	75	3	—	1·7	2·3	10	Favarger & Metzger (1952)
Deuterium-labelled (3·0 g.) in fat (60 g.)	1	80	3	—	0·7	2·3	9	Favarger & Metzger (1952)
					(%)	(%)		
Tritium-labelled (0·86 g.) in fat (85 g.)	1	—	3 7	— —	66 87	34 13	— —	Biggs *et al.* (1952)
Tritium-labelled (0·69 g.) in fat (20 g.)	1	—	3 7	— —	33 57	67 43	— —	Biggs *et al.* (1952)

Absorption of cholesterol

There are few reported experiments, using the balance method, on the absorption of cholesterol in man. In Table 10 are given values collected from the literature. When expressed on a kg. body wt./day basis the values obtained by Favarger & Metzger (1952) are similar to those obtained in this study. Bürger & Winterseel (1929) do not give the weights of their subjects, but calculating on a 70 kg. basis results of a similar order are obtained, although the percentage absorption found by these two groups of investigators were higher than ob-

Holmes (1917) found that feeding of egg-yolk fat equivalent to fourteen eggs per day for 3 days to six volunteers gave a digestibility coefficient for fat of 94%, which compared with values of 97% for butter and 93% for beef fat. Long-continued consumption of eggs (as egg yolk) gives rise to increased levels of plasma cholesterol (Okey & Stewart, 1933; Messinger, Porosowska & Steele, 1950), and a massive dose taken over a short period causes a transient rise (Collen, De Kruif & Geier, 1949). It is remarkable that under such favourable conditions there is not a much more marked absorption.

The level of cholesterol in the plasma of man is the highest of all mammals, a fact which is difficult to explain. The reported half-life time of plasma cholesterol in man is 8–11 days (see Fukushima & Rosenfeld, 1954), which indicates a turnover approaching that in experimental animals.

The picture given here is one of low absorption and of a slow metabolism as compared with the rat (Cook *et al.* 1955). Further experiments are needed to elucidate the fate of cholesterol in man.

SUMMARY

1. Cholesterol in the form of crystalline cholesterol (9·8 g.) and as egg cholesterol (6·9 g.) was taken by a human subject, and the course of intestinal excretion studied over 3 and 4 days respectively.

2. Analyses were made of the ether–ethanol extracts of the faeces for unsaponifiable matter, for free, total and saturated sterols and for steam-volatile and ether-extractable acids.

3. The crystalline cholesterol was absorbed to the extent of about 15% or about 8 mg./kg. body wt./day, and the egg sterol was about 60% absorbed or about 15 mg./kg. body wt./day. A transient elevation of the plasma level of cholesterol as esters occurred with the egg sterol.

4. The neutral fraction of the faecal extract was fractionated by chromatography and the main fractions were analysed.

5. The steam-volatile acids of the faecal extracts from the normal diet and after ingestion of crystalline cholesterol were analysed and shown to consist mainly of acetic and butyric acids.

6. The ether-extractable acids were fractionated by treatment with light petrolum. The soluble portion was separated into 'solid' and 'liquid' acid fractions which were analysed. The solid acid fractions were separated by reversed-phase chromatography.

7. The course of faecal excretion of lipid was followed, and a review is given of the balance method for studying cholesterol absorption in man.

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β-Glucuronidase in Lymphatic Tissues

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In the course of our biochemical investigations of lymphatic tissue in normal and pathological states the enzyme β-glucuronidase has been studied in conditions of atrophy and proliferation.

In the fasting animal lymphatic tissue undergoes a very marked involution (Hammar, 1905), the weight loss beginning earlier and at a higher rate than in any other tissue (Jolly, 1914; see Dougherty, 1952, for a comprehensive review). Subsequent refeeding brings about rapid reconstitution of the tissue (Frank, Kumagai & Dougherty, 1953). Adrenalectomy causes an increase in the weight of lymphatic tissue (Dougherty, 1952). Tissues undergoing such sharp variations in weight, clearly related with rates of breakdown and proliferation of the cells, offer promising material with which to test the much discussed relationship between β-glucuronidase and the process of cell growth (Levvy, Kerr & Campbell, 1948; Kerr, Campbell & Levvy, 1949, 1950; Mills, Paul & Smith, 1953).

The present communication is concerned with measurements of the β-glucuronidase activity in lymphatic organs made on tissue undergoing both degeneration during fast and regeneration on resumption of nutrition. The effects of adrenalectomy on the enzyme have also been studied. Enzyme activities have been assessed, both in terms of tissue weight and on the basis of cell counts.

EXPERIMENTAL

Young male albino rats of the 'Italico' strain, bred in this Laboratory and weighing between 78 and 118 g., were used throughout. They were kept on a standard balanced diet (Randoin & Causeret, 1947) supplemented by green vegetables. Adrenalectomy was performed by the dorsal approach under light ether anaesthesia. Animals were killed by exsanguination. Lymphatic tissues (lymph nodes, thymus and in some experiments the spleen) were immediately dissected free of connective tissue and fat and kept at ice temperature during the collection of the rest of the tissue. As representative of the lymph nodes, those superficial to the neck, a total of six, were taken. After weighing, the tissues were dispersed in distilled water (adjusted to pH 7·4) with a glass homogenizer (Potter & Elvehjem, 1936) kept in cracked ice. The final dilution of the dispersions was 1:200 (w/v). These dispersions were stored in the cold room at 2° and were always used within 12 hr. of preparation.

Determination of β-glucuronidase activity. Recrystallized phenolphthalein glucuronide prepared biosynthetically (Fishman, Springer & Brunetti, 1948) was used as substrate. The composition of the incubated mixtures was: 0·1 ml. of 0·01 M phenolphthalein glucuronide, 0·8 ml. of 0·20 M acetate buffer, pH 5·2, and 0·1 ml. of tissue dispersion. Mixtures were incubated in a water bath at 37° for 1 hr. After addition of 1·0 ml. of glycine–NaOH–NaCl buffer (pH 12·4), followed by centrifuging, the intensity of colour was measured with a Leitz photoelectric colorimeter, using cuvettes 0·5 cm. wide, and with a filter having peak transmission at 550 mμ. Freshly prepared dilute solutions of phenolphthalein were taken as standards, and all measurements were made against substrate blanks. The determinations with enzyme were made in duplicate. Results are expressed in Glucuronidase units (G.U.), one unit being defined as the amount of enzyme which liberates 1 μg. of phenolphthalein in 1 hr. in the conditions defined. The measurements of pH were made with a glass electrode standardized with buffer at pH 6·5.

Nitrogen determinations. The total nitrogen content of dispersions was determined by the micro-Kjeldahl method; digestions were carried out in the presence of potassium persulphate, and ammonia, distilled in the Markham (1942) apparatus, was collected in 0·0143 N-H_2SO_4 for back titration, using Tashiro's indicator.

Isolation of lymphocytes. The method was based on that of Hechter & Johnson (1949). Pooled thymus glands (2–4 from normal rats, and 3–5 from fasting rats) were minced with scissors and suspended in cold 0·9% (w/v) NaCl solution. The suspension was filtered through folded sterile muslin and the lymphocytes were washed three times by centrifuging with cold saline. After being brought to the original volume with the saline a portion was diluted by addition of ten times its volume of Türk fluid in a blood white-cell pipette and counted in a Bürker camera by two observers. Smears were also made and stained according to May–Grünwald–Giemsa and Pappenheim. This showed that in both normal and fasting animals almost all the cells present were lymphocytes. These were mostly small, but some medium-size and large cells were also present. Smears from fasting animals often showed nuclear pyknosis and a cytoplasm less readily stained than in normal material.

The bulk of the preparation was again centrifuged, resuspended in the original volume of distilled water at pH 7·4 and dispersed in the homogenizer before determination of β-glucuronidase activity, which was calculated in terms of a standard count of ten million cells.

RESULTS

Variations in enzyme activity due to various factors

In preliminary experiments the optimum conditions for the assay of β-glucuronidase in lymphatic tissue were established. For this purpose homogenates from pooled neck lymph nodes were used exclusively.

Variations in activity with pH. The pH–activity curve (Fig. 1) showed two well-marked optima at pH 4·2 and 5·2, the latter being the point of highest activity. This result is substantially similar to those of Kerr *et al.* (1949) for liver and kidney. The pH chosen finally for assays was therefore 5·2.

Variation in activity with concentration of substrate. There was optimal glucuronidase activity when the final concentration of phenolphthalein glucuronide was 0·001 M, as in the assay method of Talalay, Fishman & Huggins (1946). This concentration was chosen for use in the present investigation.

Effect of enzyme concentration. In the range 0–1·25 mg. of tissue/ml. of final mixture, the activity was directly proportional to the weight of tissue (Fig. 2). Subsequent assays were set up with 0·5 mg. tissue/ml., the tissue being taken as suspensions, 0·5 % (w/v), in water.

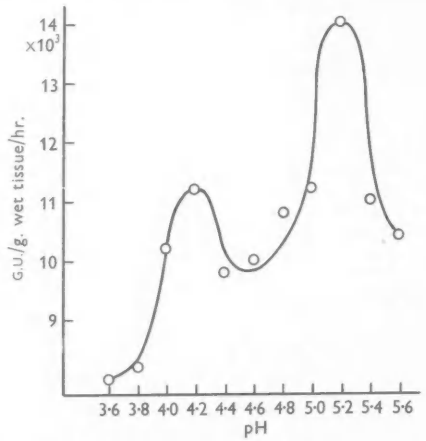

Fig. 1. Effect of pH on the liberation of phenolphthalein from 0·001 M phenolphthalein glucuronide by lymph nodes dispersions. Acetate buffers, 0·16 M. Other conditions of the assay as described in the text.

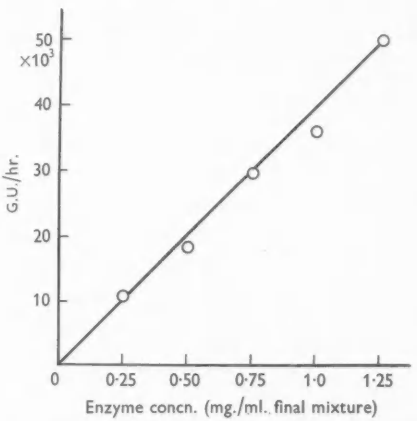

Fig. 2. Relationship between hydrolysis of phenolphthalein glucuronide and concentration of the enzyme. Standard conditions of assay as described in the text.

Table 1. *Wet weights and β-glucuronidase activities of neck lymph nodes, thymus and spleen of normal rats, of rats fasted for* 96 *hr. and of adrenalectomized rats (means* ±S.E.M.)

Condition	No. of rats	Wet wt. (mg./100 g. initial body wt.)	Wet wt. as % of wet wt. of normals	β-Glucuronidase G.U./g. wet wt.*	β-Glucuronidase G.U./mg. N	G.U./mg. N in % of the values of the normals	Total amount of enzyme per organ or pooled organs (G.U.)
Neck lymph nodes							
Normals	15	153±14	100	14 400±800	523±34†	100	2 203±221
Fasted for 96 hr.	9	71±5‡	46	22 040±1600‡	711±50‡	135	1 565±136‡
Adrenalectomized for 96 hr.	7	155±8	101	16 280±1480	569±76	108	2 523±209
Thymus							
Normals	14	195±14	100	5 920±340	195±4†	100	1 155±136
Fasted for 96 hr.	8	56±8‡	29	16 400±1800‡	449±47†‡	270	918±103
Adrenalectomized for 96 hr.	7	263±19‡	135	5 800±720	178±14	91	1 526±83
Spleen							
Normals	7	857±178	100	15 200±860	—	—	12 986±1596
Fasted for 96 hr.	10	255±44‡	30	42 800±1140‡	—	—	10 914±1418
Adrenalectomized for 72 hr.	5	756±145	88	20 200±900‡	—	—	15 271±605

* For definition of G.U. see text. † Seven rats only.
‡ Difference with normals statistically significant ($P<0·05$; Student's *t* test).

Effect of incubation period. Hydrolysis proceeded linearly with time over a period of at least 2 hr. The standard incubation period decided upon was of 1 hr.

β-Glucuronidase activity in lymphatic tissue after 96 hr. fasting and after bilateral adrenalectomy

In a preliminary survey, levels of β-glucuronidase were determined in two experimental conditions such that the weight of lymphatic tissue had undergone variations in opposite directions. In the first place a condition of fasting was enforced. This caused a rapid loss in weight, the maximum effect being observed in the thymus, which diminished to about 25 % of its normal weight after 96 hr. In the second case adrenalectomy was performed; this resulted in an increase in weight, also particularly evident in the thymus gland, which went up by 35 % in 4 days from operation.

Corresponding with decreased weight of tissues, a clear increase in β-glucuronidase activity was observed. The effect was greatest in thymus and spleen, where the relative loss of weight was greatest. On the other hand, 4 days after adrenalectomy, a significant increase in β-glucuronidase activity was apparent only in the spleen, corresponding with a slight loss in weight. In the lymph nodes and thymus there were slight and not significant variations in the enzyme, although the weights of the thymus had increased markedly in comparison with the normal.

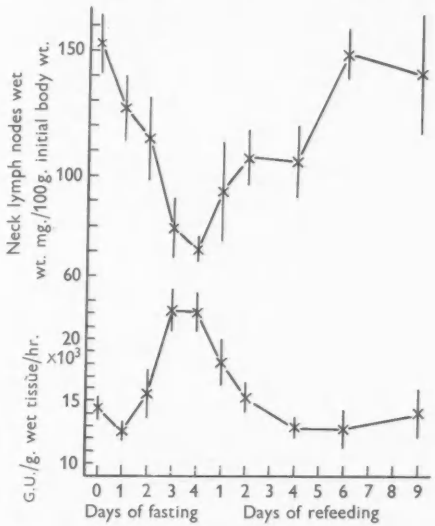

Fig. 3. Effect of fasting and refeeding of rats on the weight and glucuronidase activity of neck lymph nodes. Wet weight (mg./100 g. body weight at the beginning of the experiment): upper curve; β-glucuronidase activity (G.U./g. wet weight of tissue): lower curve. The vertical segments correspond to 2 × S.E.M. Each experimental point corresponds to the mean of from five to eleven individual results.

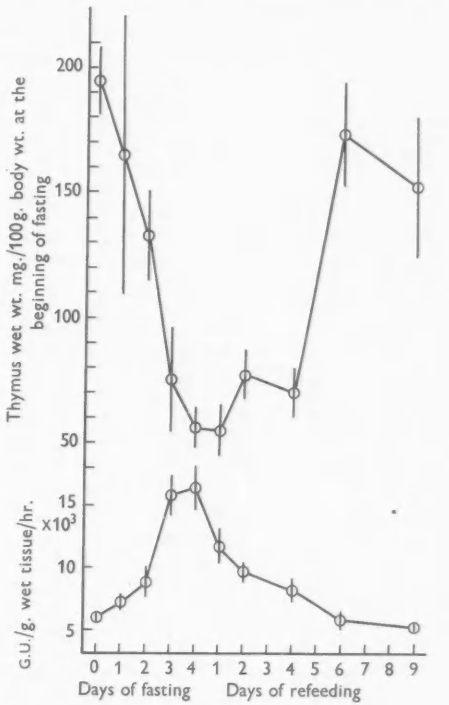

Fig. 4. Effect of fasting and refeeding of rats on the weight and glucuronidase activity of thymus glands. Wet weight (mg./100 g. body weight at the beginning of the experiment): upper curve; β-glucuronidase activity (G.U./g. wet weight of tissue): lower curve. The vertical segments correspond to 2 × S.E.M. Each experimental point corresponds to the mean of from five to eleven individual results.

These results seemed to establish a link between decrease in weight of lymphatic tissue and increase in the activity of β-glucuronidase, whereas increase in weight seemed not to be accompanied by any clear variation in the activity of the enzyme. It was therefore deemed necessary to follow closely the behaviour of glucuronidase in correlation with the variations in weight, both positive and negative, of the tissues. Such conditions could be easily obtained by fasting the animals for 4 days, so that

lymphatic tissue decreased sharply in weight, and then refeeding them to bring about reconstitution of the tissue. Figs. 3 and 4 show the variations in weight and in enzyme activity under these conditions for lymph nodes and thymus glands. It is apparent that β-glucuronidase activity increased in both thymus and lymph nodes during the period in which these organs were undergoing loss in weight, and that it began to fall rapidly on refeeding.

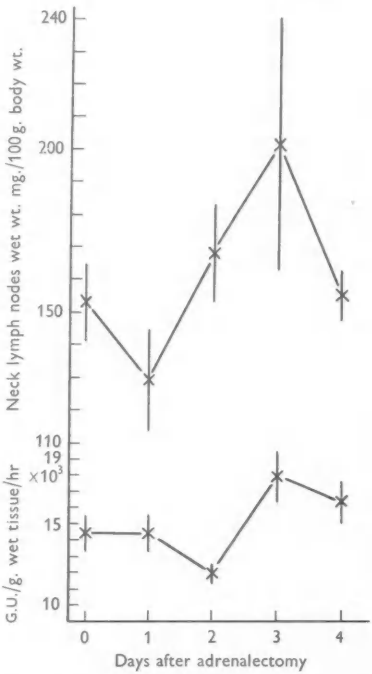

Fig. 5. Wet weight (upper curve) and β-glucuronidase activity (lower curve) of neck lymph nodes after bilateral adrenalectomy. Each experimental point corresponds to the mean of from six to eleven experimental results. The vertical segments correspond to 2 × S.E.M.

Further data on the relationship between the weight of lymphatic tissue and glucuronidase activity were obtained in adrenalectomized rats (Figs. 5, 6). Whereas in the lymph nodes glucuronidase activity was slightly increased in correlation with a marked increase in weight, in thymus a very pronounced increase in weight was accompanied by only small changes in the activity of the enzyme.

β-Glucuronidase content of isolated lymphocytes

Since the specific activity of atrophic lymphatic tissues was found to be elevated, while the total amount of enzyme in the organs was if anything diminished (Table 1), it could be supposed that

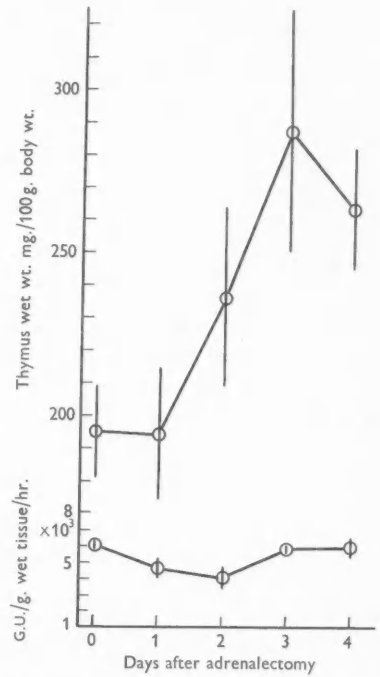

Fig. 6. Wet weight (upper curve) and β-glucuronidase activity (lower curve) of thymus after bilateral adrenalectomy. Each experimental point corresponds to the mean of from six to eleven experimental results. The vertical segments correspond to 2 × S.E.M.

Table 2. *β-Glucuronidase activity of lymphocytes isolated from the thymus* (G.U./10 *million cells*)

	Expt. no. 1	Expt. no. 2	Expt. no. 3
Normals	8 640	24 000	30 800
Fasted for 96 hr.	18 200	39 200	38 800

fasting resulted in the preferential mobilization of enzymically inactive material. This hypothesis was tested by determinations of glucuronidase activities of lymphocytes isolated from thymus glands of normal and of fasted animals. The results (Table 2) show that during the fast an actual increase in the activity of glucuronidase per cell takes place and,

further, demonstrate that lymphocytes contribute to the increases found in the tissues. Without further evidence it is not possible to assess the part played by other elements such as reticular cells and stroma, which are relatively increased as a result of fasting.

DISCUSSION

The main result arising from the experiments reported here is that in lymphatic tissues the activity of glucuronidase increases considerably under conditions which bring about sharp decrease in weight of the tissue; under conditions in which the weight increases, β-glucuronidase levels, if previously elevated as a result of experimental intervention, return to normal values, and if previously normal show no significant change. This pattern of variation follows the same course whether activity is expressed as an enzyme:wet weight ratio, or as activity/unit weight of nitrogen (Table 1). In view of the elevated enzymic activity per cell found during fasting, it was thought that this could be due to a decrease in the content of some inhibitor normally present in the tissues (which was actually found in various organs of the rat, including the spleen, by Walker & Levvy, 1953). Experiments set up to test this hypothesis (Table 3) were not convincing, as even when four parts of the normal material were used for each part of the starved tissue the inhibition found ranged only from 14 to 21 %.

Table 3. *Influence of mixtures in various proportions of lymph nodes dispersions from normal and fasted rats on the hydrolysis of phenolphthalein glucuronide*

Mixtures of tissue dispersions 0·2 ml./ml. of incubated mixture. Acetate buffer 0·7 ml. For other conditions of assay see text.

Parts of dispersions		β-Glucuronidase activity (G.U./g. of tissue)		
From normals	From fasted	Found	Theoretical*	Inhibition (%)
		Expt. no. 1		
2·5	2·5	18 000	23 600	24
4	1	14 700	18 600	21
1	4	21 900	23 500	7
		Expt. no. 2		
2·5	2·5	16 700	19 200	13
4	1	14 300	16 700	14
1	4	18 800	19 800	5

* Sum of the activities found when each portion of enzyme was incubated separately.

Histological examination of lymphatic tissue revealed that during the period when the weight is declining as a result of fasting there was loss of lymphocytes, leaving a relative predominance of reticulo-endothelial cells. Mitoses, while not disappearing, were much less numerous than in normal conditions. These observations are in agreement with those reported by Dustin (1923), Undritz (1924), Schillaci (1928) and Frank *et al.* (1953). They show that the increase of glucuronidase activity in lymphatic tissue takes place during the period of reduced cellular proliferation, i.e. when the cells undergo atrophy and reduction in number. When, on the other hand, under the influence of refeeding or adrenalectomy, a marked increase in the lymphocyte count takes place, the level of enzyme activity does not vary unless an increase has been caused previously; in such a case a rapid fall in activity is observed.

The present findings are not compatible with the hypothesis that increase in β-glucuronidase is closely related to the proliferation of cells (Levvy *et al.* 1948; Kerr *et al.* 1949, 1950). This concept is based mainly on the increase in enzyme activity of liver and kidney found after the administration of various toxic substances. The results of these authors could also be interpreted as indicating an increase in β-glucuronidase coinciding with cellular damage (Mills *et al.* 1953). Other reports in the literature indicate a lack of correlation between variations in β-glucuronidase levels and cell proliferation. Some of these are concerned with the influence of hormones on the genital tract (Harris & Cohen, 1951; Knobil, 1952; Beyler & Szego, 1954), others with the behaviour of peripheral nerve in Wallerian degeneration (Hollinger & Rossiter, 1952), and yet others with the enzyme activity in rat liver at various stages of growth (Mills *et al.* 1953). Further examination of the literature discloses some cases in which increases in the activity of the enzyme show positive correlation with atrophy or involution of the tissue. Knobil (1953) observed a significant increase of glucuronidase activity in adrenal glands during cortical atrophy following hypophysectomy. Fishman & Farmelant (1953) found an increased activity in the seminal vesicles of castrated mice;

which was reversed by treatment with testosterone. Greenbaum & Greenwood (1954) observed an increase in glucuronidase activity during the period of involution of the mammary gland after lactation.

The results of Fishman & Anlyan (1947), Fishman, Anlyan & Gordon (1947) and of Odell & Burt (1949) on the elevated level of glucuronidase activity in tumours (see, however, Mills & Smith, 1951, who did not find an increase in experimental hepatoma) would not conflict with the hypothesis correlating the increase in enzyme activity with regression processes in tissues, since zones of proliferation and of regression and necrosis are intimately interwoven in malignant tumours. On the other hand, the data of Fishman & Fishman (1944) and Fishman (1947) on the increase of β-glucuronidase activity in the uterus under the influence of oestradiol, and those of Riotton & Fishman (1953) and of Fishman & Farmelant (1953) on changes in kidney brought about by testosterone, cannot be reconciled with such an hypothesis. In the present study an attempt was made to determine whether injections of corticosteroids would produce any changes in the enzyme levels in lymphatic tissue. However, the result was negative; a single injection of cortisone acetate (Cortone, Merck), 6·2 or 12·5 mg./rat, did not induce any appreciable modification in the weights of the organs or of the activity of the enzyme in them.

Little can be said at present on the mechanism by which β-glucuronidase activity increases during atrophy. Further work aimed at clarifying this point is in progress.

SUMMARY

1. Variations in the levels of β-glucuronidase activity of rat lymphatic tissue have been followed, with phenolphthalein glucuronide as substrate, during periods of (i) loss in weight of the tissues caused by fasting, (ii) reconstitution by resumed feeding, and (iii) increase in weight which takes place after adrenalectomy.

2. β-Glucuronidase activity increased, while the weight of the tissue decreased. When the tissue increased in weight the enzyme level, if previously raised, fell to a normal value, or if already normal showed no significant variation.

3. Significant increases in the levels of enzyme, expressed as activity per cell, take place when mitoses are inhibited, and lymphocytes undergo atrophy and disappear.

4. The results are not compatible with the hypothesis that increase of β-glucuronidase is correlated with cell growth; they show that the increase in enzyme activity, at least in some cases, coincides with atrophy and involution of the tissue.

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On the Mechanism of Fluid Exchange of Tissues *in vitro*

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There have been many studies in which hydration of mammalian tissues has been determined after incubation in saline media under varying conditions of metabolism (reviewed by Robinson, 1953). The results indicate that when metabolism is inhibited, various tissues swell in saline media unless the osmotic concentration is greater than about twice that of extracellular fluid (Opie, 1949; Robinson, 1950; Aebi, 1953). When tissue metabolism is active the changes in hydration are much less (Stern, Eggleston, Hems & Krebs, 1949; Robinson, 1950; Aebi, 1952*b*). These findings have been interpreted as evidence that the intracellular fluids are normally hypertonic with respect to their extracellular fluid environment and that this hypertonicity is maintained by active metabolic processes (Robinson, 1950, 1953; Robinson & McCance, 1952; Bartley, Davies & Krebs, 1954).

The validity of such an interpretation is entirely dependent upon the assumption that net movements only of water occur across the cell membranes during such experiments *in vitro*. If net movements of osmotically active substances occur as well, observed changes in hydration cannot be used as evidence for the existence of differences between the total solute concentrations of intra- and extra-cellular fluids of metabolizing tissues. The present results demonstrate that large net shifts of ions do in fact occur across cell membranes when metabolism is inhibited. A different explanation of the changes in tissue hydration is presented which is based upon the known dependence on metabolism of ionic gradients in tissues.

EXPERIMENTAL

Studies were made on slices of guinea-pig kidney cortex, rat cerebral cortex and rat liver. The outermost slice of tissue was rejected. Slices approximately 0·3 mm. thick were spread on hardened filter paper (Whatman no. 54) to remove surface blood, urine and cellular debris. Kidney cortex slices were incubated in a bicarbonate–saline solution similar to medium III of Krebs (1950) but without the addition of the organic substrates. To each 2 ml. of medium gassed with O_2+CO_2 (95:5) was added 0·1–0·2 g. of tissue. The flasks were kept at 0° to inhibit metabolism and were shaken in a Dubnoff Metabolic Shaking Incubator (Precision Scientific Co., Chicago, U.S.A.) for 30 min. The slices were reblotted and then weighed.

Slices of liver and cerebral cortex were similarly treated except that the medium used was a mixture of sodium and potassium chlorides containing 160 m-equiv. of Na and 5 m-equiv. of K/l. Because of their friability slices of cerebral cortex could not be blotted on filter paper. Excess of moisture was removed by repeatedly touching the slices against a clean glass plate. The control slices were similarly dried after momentary immersion in the incubating medium.

The water content of the slices was obtained as the difference between their weights before and after drying overnight at 105°. The tissue electrolyte determinations were made on an extract obtained by digesting the tissue specimen for at least 24 hr. at room temperature in 5·0 ml. of 0·1 N-HNO_3. This method of digestion avoids loss of chloride by volatilization or by conversion into organic chloro compounds (Shenk, 1954). Chloride of tissues and media was analysed by the method of Sanderson (1952). Na and K concentrations of tissue and supernatant fluid were determined with a Beckman DU Spectrophotometer with flame-photometer attachment. Portions of the dilute acid extract or supernatant fluid were further diluted to give final concentrations of Na and K of approximately 0·15 m-equiv./l. Na and K were added to yield final concentrations of 5 m-equiv./l. in the samples analysed for potassium and sodium respectively.

RESULTS

Table 1 shows the effects on the water, sodium, chloride and potassium contents per kilogram of tissue solids of incubating fresh slices of guinea-pig kidney cortex at 0° for 30–50 min. The changes in water and electrolytes are all statistically significant. The tissue swelling and reciprocal changes in sodium and potassium content have been extensively discussed by previous workers, but of special interest is the large gain in tissue chloride that occurred. For the increase of 0·94 l. of water by the swollen tissue a concomitant increase of 145 m-equiv. of chloride took place. The chloride concentration in the increment of water gained may be said to equal 154 m-equiv./l. whereas the incubation medium had a concentration of chloride of from 130 to 140 m-equiv./l. It may be further calculated that some 145–170 m-equiv. of sodium entered the tissue in accompaniment with this

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gain in tissue chloride, the amount depending upon the degree to which penetration of other anions beside chloride (phosphate and bicarbonate) of the medium took place. Of the 229 m-equiv. of sodium that entered the tissue there thus remains some 60–85 m-equiv. which entered the tissue in net exchange for potassium. Exact equality between this increment of sodium gained and the potassium lost by the tissue cannot be expected because of the likelihood of some loss of diffusible anions from the cells. It is apparent from these results that the increase of tissue sodium considerably exceeds the loss of tissue potassium, and that the swelling process may be considered as a gain by the tissue of approximately isotonic medium.

140 m-equiv./l. Thus the tissue swelling may be said to result from an entry of approximately isotonic medium into the tissue when metabolism is interfered with by any of the means tested.

Table 3 shows that the swelling of cerebral cortex and liver slices *in vitro* involves similar ion movements into these tissues. The calculated chloride concentration in the water gained was 225 and 231 m-equiv./l. for cerebral cortex and liver slices respectively. The concentration of chloride in the medium was 165 m-equiv./l. The discrepancy between these values is in the same direction but larger than that seen in results presented so far and will be discussed below. After subtracting the equivalent quantity of sodium which entered the

Table 1. *Changes in the sodium, potassium, chloride and water contents of guinea-pig kidney cortex slices on incubation at 0°*

Slices of tissue (0·1–0·2 g.) incubated 30–50 min. in 2·0 ml. of bicarbonate–saline, pH 7·4, gas phase $O_2 + CO_2$ (95:5). Mean values ± S.E.

	No. of samples	Dry wt. (%)	Water per kg. dry solids (kg.)	m-equiv. per kg. dry solids		
				Na	Cl	K
(*a*) Before incubation	14	23·7±0·35	3·22±0·04	350±16	272± 7	365± 8
(*b*) After incubation	14	19·4±0·29	4·16±0·05	579±18	417±17	240± 7
b − *a*	—	—	+0·94±0·06	+229±24	+145±18	−125±11

Table 2. *Changes in the sodium, potassium, chloride and water contents of guinea-pig kidney cortex slices on incubation at 37°, at 0°, with 0·2 mM 2:4-dinitrophenol (DNP), and anaerobically, mean values* ± S.E.

Data recalculated from Whittam (1954).

	No. of samples	Temp. at incubation °	Aerobic or anaerobic	Dry wt. (%)	Water/kg. dry solids (kg.)	m-equiv. per kg. dry solids		
						Na	Cl	K
(*a*) Before incubation	16	—	—	22·3±0·2	3·49±0·02	284± 9	229±36	342±4
(*b*) After incubation	23	37	Aerobic	20·3±0·3	3·93±0·05	444± 9	277±28	370±8
(*c*) In cold	12	0	Aerobic	17·0±0·5	4·89±0·12	659±17	460±34	182±4
(*d*) With DNP	—	37	Aerobic	17·0	4·89	730	454	204
(*e*) Anaerobic	18	37	Anaerobic	17·3±0·3	4·78±0·07	660±13	455±28	159±6
b − *a**	—	—	—	—	+0·44±0·05	+160±13	+48±46	+28±9
c − *a*	—	—	—	—	+1·40±0·14	+375±19	+231±50	−160±6
d − *a*	—	—	—	—	+1·40	+446	+225	−138
e − *a*	—	—	—	—	+1·29±0·08	+376±16	+226±46	−183±7

* The increase in sodium found on incubation at 37° with α-oxoglutarate as substrate was shown by Whittam & Davies (1953) to be correlated with an accumulation of substrate in the tissue. This accumulation of sodium oxoglutarate in the tissue may account for the small increase in tissue hydration without significant increase in tissue chloride.

Table 2 shows similar results which have been obtained by recalculation of data from Whittam (1954). The effects of oxygen lack and of 2:4-dinitrophenol, as well as of incubation at 0°, are shown. Significant increases in tissue water, sodium and chloride with loss of potassium are again noted (*c* − *a*, *d* − *a*, and *e* − *a*). The gains in tissue chloride were sufficient to yield calculated chloride concentrations in the increments of water of 165, 160 and 175 m-equiv./l. with chilling, 2:4-dinitrophenol, and anaerobiosis respectively. The chloride concentration of the medium was 130–

tissue with chloride, there remain 179 and 120 m-equiv. to exchange for the observed potassium loss of 257 and 178 m-equiv. from cerebral cortex and liver slices respectively. As chloride was the only anion in the medium, 78 (257 − 179) and 58 (178 − 120) m-equiv. are estimates of the loss of diffusible anions from the cells of cerebral cortex and liver slices respectively.

Deyrup (1953*a*) has noted that tissue slices show very little change in water content when immersed in sucrose solutions approximately isotonic with extracellular fluid. Table 4, *b* − *a*, confirms this

Table 3. *Changes in the sodium, chloride, potassium and water contents of rat cerebral cortex and liver on incubation at 0°*

Slices of tissue (0·1–0·2 g.) incubated 30 min. at 0° in 3·0 ml. of 160 m-equiv. NaCl and 5 m-equiv. KCl per l. Mean values ± S.E.

	No. of samples	Dry wt. (%)	Water/kg. dry solids (kg.)	m-equiv. per kg. dry solids		
				Na	Cl	K
Rat cerebral cortex						
(*a*) Before incubation	6	22·7 ± 0·3	3·40 ± 0·03	312 ± 19	219 ± 7	451 ± 17
(*b*) After incubation	6	17·5 ± 0·2	4·72 ± 0·04	788 ± 23	516 ± 13	194 ± 9
b − *a*	—	—	+1·32 ± 0·05	+476 ± 30	+297 ± 15	−257 ± 19
Rat liver						
(*c*) Before incubation	6	30·5 ± 0·6	2·28 ± 0·03	114 ± 14	121 ± 4	345 ± 6
(*d*) After incubation	6	23·1 ± 0·03	3·33 ± 0·03	477 ± 34	364 ± 20	167 ± 12
d − *c*	—	—	+1·05 ± 0·04	+363 ± 37	+243 ± 20	−178 ± 13

Table 4. *Changes in sodium chloride, potassium and water contents of guinea-pig kidney cortex slices on incubation in sucrose-containing medium*

Slices of tissue (0·1–0·2 g.) incubated 30–180 min. in 0·25 M sucrose at 0°. Mean values ± S.E.

	No. of samples	Dry wt. (%)	Water/kg. dry solids (kg.)	m-equiv. per kg. dry solids		
				Na	Cl	K
(*a*) Before incubation	14	23·7 ± 0·35	3·22 ± 0·04	350 ± 16	272 ± 7	365 ± 8
(*b*) In 0·25 M sucrose	4	23·5 ± 0·73	3·25 ± 0·08	155 ± 18	46 ± 16	179 ± 4
b − *a*	—	—	+0·03 ± 0·09	−195 ± 24	−226 ± 17	−186 ± 9

observation; the mean dry weight equals that of the control. However, measurement revealed marked losses of sodium, potassium and chloride from the tissue. These large losses of osmotically active tissue constituents indicate that though the tissue may be in osmotic equilibrium with its medium after incubation in such a way as to preserve the normal state of tissue hydration, this finding bears little relationship to the initial osmotic state of the tissue.

DISCUSSION

In 1951 Mudge (1951*b*) presented results from studies *in vitro* showing that the swelling produced by a variety of inhibitors of metabolism was associated with an uptake of ions as well as of water by the swollen tissue. The present results are fully in accord with his conclusion that: 'Depression of metabolic activity is, therefore, associated with an isosmotic increase in cellular hydration.'

The gain in tissue water and solute in the present experiments is probably shared by both the intra- and extra-cellular phases of the tissue. Thus, Robinson (1950) found by measurement of the inulin and sucrose spaces in slices of rat renal cortex that the 'extracellular' water remains an approximately constant fraction (26%) of the weight of the slices whether tissue hydration is normal or increased. This finding has been confirmed with slices of guinea-pig cortex incubated aerobically at 0° (Whittam, 1954).

Several workers have noted a loss of tissue solids during incubation *in vitro* (Robinson, 1949; Aebi, 1952*a*). This may amount to 8–18% of the nitrogen content of liver slices (Aebi, 1952*a*). Others have demonstrated, however, that re-incubation of swollen tissue in media favourable to metabolic processes results in a return of tissue hydration and electrolyte composition toward control levels (Terner, Eggleston & Krebs, 1950; Krebs, Eggleston & Terner, 1951; Robinson, 1950; Mudge, 1951*a*; Deyrup, 1953*b*; Whittam & Davies, 1953). Hence the loss of tissue solids during incubation does not appear to affect the present results.

The finding that tissues gain solute as well as water invalidates the argument that the process of swelling involves a movement only of water into cells in response to a high intracellular osmotic pressure. Another explanation to account for changes in tissue hydration is required.

Cell	Medium
A^{n-}	Na_o^+
K_i^+	Cl_o^-
Cl_i^-	K_o^+

Fig. 1.

As shown in Fig. 1, a cell and its medium may be considered to constitute a 'double' Donnan system in which the cell membrane separates a non-diffusible intracellular anion, A^{n-} with n negative charges, and an extracellular cation, Na^+, excluded from the cell. The osmotic effects of these two ions will thus counterbalance each other, and the

normal cell volume will be maintained. The extracellular position of Na^+ is, however, dependent upon a supply of metabolic energy. When metabolism is disturbed Na^+ enters the cells. It will be shown that this must result, as well, in an entry of extracellular anions into the cell, a rise of intracellular osmotic pressure and swelling of the cell.

The distribution of diffusible ions is described by:

$$[K^+]_i\,[Cl^-]_i = [K^+]_o\,[Cl^-]_o, \quad (1)$$

and

$$[K^+]_i = [Cl^-]_i + n[A^{n-}]. \quad (2)$$

Then

$$([Cl^-]_i + n[A^{n-}])\,[Cl^-]_i = [K^+]_o\,[Cl^-]_o, \quad (3)$$

or

$$[Cl^-]^2 + n[A^{n-}]\,[Cl^-]_i = [K^+]_o\,[Cl^-]_o,$$

where [ ] indicate concentrations, activity coefficients being assumed to be unity throughout, and $_i$ and $_o$ refer to intra- and extra-cellular concentrations respectively.

If metabolism is disturbed so that Na^+ which diffuses into the cell is no longer actively extruded, the distribution of diffusible ions becomes:

$$\frac{[K^+]_i}{[K^+]_o} = \frac{[Na^+]_i}{[Na^+]_o} = \frac{[Cl^-]_o}{[Cl^-]_i}, \quad (4)$$

and

$$[K^+]_i + [Na^+]_i = [Cl^-]_i + n[A^{n-}]. \quad (5)$$

Substituting into (5) the values of $[K^+]_i$ and $[Na^+]_i$ obtained from (4)

$$[Cl^-]_i^2 + n[A^{n-}]\,[Cl^-]_i = [K^+]_o\,[Cl^-]_o + [Na^+]_o\,[Cl^-]_o. \quad (6)$$

The left-hand terms of equations (3) and (6) contain the variable, $[Cl^-]_i$, of interest, and a constant, $n[A^{n-}]$. The right-hand terms can all be considered constants if the same medium is used because of the relatively large volume of medium. A comparison of equations (3) and (6) indicates that the value of $[Cl^-]_i$ must be greater in the latter.

The osmotic pressure difference, ΔP, between cell and medium in this simple example may be expressed:

$$\Delta P = \boldsymbol{RT}\,(\Sigma \text{ intracellular ions} - \Sigma \text{ extracellular ions})$$

assuming osmotic coefficients of unity for each ion species in the hypothetical system. As derived in Appendix I, with sodium excluded from the cell,

$$\Delta P_1 = \boldsymbol{RT}\left[[A^{n-}] + \sqrt{\{n^2[A^{n-}]^2 + 4[K^+]_o\,[Cl^-]_o\}} - 2[Cl^-]_o\right] \quad (7);$$

whereas with sodium freely entering the cell,

$$\Delta P_2 = \boldsymbol{RT}\left[[A^{n-}] + \sqrt{\{n^2[A^{n-}]^2 + 4([K^+]_o\,[Cl^-]_o + [Na^+]_o\,[Cl^-]_o)\}} - 2[Cl^-]_o\right]. \quad (8)$$

Inspection of equations (7) and (8), in which all terms are considered constants when the external medium is the same, indicates that $\Delta P_2 > \Delta P_1$. This increase in ΔP_2, which is a necessary consequence of the inability of the cells to extrude sodium actively when metabolic processes are inhibited, will result in a swelling or increased hydration of the cell if its membrane is distensible. Re-establishment of normal metabolism, by causing extrusion of sodium from cells, will secondarily reduce the tissue swelling. Such a mechanism is in accord with the recently proposed hypothesis of Wilson (1954).

For purposes of the present argument it is immaterial whether the cell membrane is assumed impermeable to sodium or whether the membrane is permeable, but the accumulation of sodium in the cell is actually prevented by active extrusion of sodium from the cells. Abundant evidence (see Ussing, 1952; Steinbach, 1952; Hodgkin & Keynes, 1955) supports the latter alternative. The prevention of sodium accumulation as a consequence of an active uptake of potassium or of its selective intracellular accumulation (Ling, 1952, 1955) would likewise be compatible with the present concept. The quantity of sodium apparently normally present in the cell can be ignored for purposes of simplicity.

This explanation of the observed entry of chloride into tissues whose metabolism has been disturbed has been based on the currently accepted view that cations are the actively transported ions. Should it be shown alternatively that chloride is actively extruded from cells its accumulation in tissues would be a direct consequence of a disturbed metabolism.

The present hypothesis is intended to afford only a qualitative description of events as they actually occur in tissues. Much more information about compartment sizes, distribution of ions and ionic activities in tissues will be necessary before any quantitatively satisfactory formulation of this problem can be achieved. Also, if the osmotic pressure of the medium in which the tissue is incubated is produced by solute (e.g. by monosaccharides) other than the customary ions, the tissue swelling will then obviously depend on the degree to which this solute penetrates the cells.

In 1907 Lillie demonstrated that the osmotic pressure exerted by a given concentration of protein decreases with increasing concentration of neutral salts in the medium. It is theoretically possible therefore (see Appendix II) that swelling of tissues may be prevented, even when metabolism is inhibited, by placing them in hypertonic salt solutions. This is exactly what Opie (1949), Robinson (1950) and Aebi (1953) have found experimentally. From the above development it is apparent, however, that this finding does not justify the claim that the high concentrations of such media represent the true total solute concentration within metabolizing cells.

The present results are compatible with the view that the increase in tissue sodium content which has been repeatedly observed under conditions of impaired cellular metabolism is divisible into at

least two components, an ion exchange of extracellular sodium with intracellular potassium and a net movement of sodium into cells together with chloride. The much larger magnitude of the increase in tissue sodium content than of the reciprocal decrease in tissue potassium is thus accounted for. This large discrepancy between sodium gained and potassium lost is easily overlooked if the changes in tissue composition are expressed per unit weight of wet tissue rather than per unit weight of tissue solids. Thus Mudge (1951*a*) found a loss of potassium of 123 and 195 m-equiv. compared with a gain of sodium of 292 and 291 m-equiv./kg. tissue solids in his initial non-leached and leached slices of rabbit kidney cortex, respectively, as compared with the fresh tissue control. In spite of the large discrepancies between quantities of sodium and potassium gained and lost by the swollen tissue it was noted that, when expressed per kg. of wet tissue: 'Within the limits of the experimental error the changes in tissue Na were the reciprocal of K and the sum of Na and K remained constant.' Whittam & Davies (1953) likewise present figures showing reciprocal and approximately equal changes in wet tissue sodium and potassium content, although the recalculation of their data in Table 2 of this paper clearly reveals the much larger increase of sodium than loss of potassium in their study. It is apparent that if expressed on a wet tissue (or tissue water) basis, a simple addition or subtraction of an isotonic solution of a sodium salt to a tissue will produce reciprocal changes in tissue potassium without any potassium necessarily being lost or gained by the tissue.

from the medium, largely sodium chloride, to return the cells to their pre-incubation level. As only swelling in excess of the pre-incubation state of hydration has been considered in the calculations, it would be expected that the chloride uptake by the swollen tissues would represent more than simply an isotonic increase of chloride in the increment of water gained. In this respect, the quantity of anions lost in accompaniment with potassium from the cells during incubation can be calculated from Table 3, in which experiments chloride was the sole anion of the medium. As stated in Results, this loss of intracellular anion per kg. of dry solids of cerebral cortex and liver was 78 and 58 m-equiv. respectively. The corresponding excess of chloride above an isotonic entry was 80, i.e. $(225-165)\times 1{\cdot}32$, and 69, i.e. $(231-165)\times 1{\cdot}05$, m-equiv. respectively. The correspondence of these values is sufficiently close, if the anions lost were univalent, to lend support to this argument.

It has been frequently stated that the sum of intracellular sodium and potassium concentrations in mammalian tissues considerably exceeds their combined concentrations in the medium or extracellular fluid (Elkinton, Winkler & Danowski, 1944; Whittam & Davies, 1953; Bartley *et al.* 1954). Though this finding is still cited occasionally as evidence for a higher intracellular than extracellular osmotic pressure, the realization is increasing that this may simply be a necessary requirement to maintain osmotic equality between the two fluids as a consequence of a considerable quantity of polyvalent anion of high molecular weight within the cells (Mudge, 1953). Assuming

Table 5. *Comparison of the calculated chloride concentration in the increment of water gained during tissue swelling with the estimated chloride concentration of the medium*

Data from Tables 1–4.

Tissue	No. of comparisons	Calc. Cl concn. in water (m-equiv./l.)	Cl concn. of medium (m-equiv./l.)	Difference (m-equiv./l.)
(1) Kidney cortex (Table 1)	14	154	135	19
(Table 2)	—	166	135	31
(2) Cerebral cortex	6	225	165	60
(3) Liver	6	231	165	66

As noted in Results and Table 5, the calculated chloride concentration in the increment of water gained by the swollen tissues was greater than the concentration of chloride of the medium. This finding is probably a necessary consequence of the manner in which the control data were obtained. Thus unincubated fresh tissue slices were compared with slices placed in media into which they might be expected to lose solute. Any loss of intracellular, osmotically active solute from the cells during incubation would have to be replaced by solute an extracellular volume of 26 % of the wet weight of the tissue (Robinson, 1950), the values of Table 1 can be used to calculate an 'intracellular' concentration of sodium plus potassium before incubation of 256 m-equiv. which decreased to 207 m-equiv./l. of intracellular water after metabolism was inhibited although the sodium plus potassium concentration of the medium remained constant at approximately 160 m-equiv./l. This decline in the gradient of intracellular to extracellular cation concentrations need not signify a fall in

intracellular total solute concentration consequent upon inhibition of metabolic processes. As Mudge pointed out (1951*b*) such a drop would be expected from the entrance of univalent anions into the cell (see Appendix III).

Stern *et al.* (1949) were the first to observe the dependence of tissue hydration on cellular respiration. Their observation has been repeatedly interpreted since as evidence for an active transport of water by cells. Without proving or disproving the possibility that primary active transport of water is a common functional property of cells (mammalian renal tubular cells, at least, would seem to be capable of this task) the present paper emphasizes that the changes in hydration which may be observed on incubation *in vitro* can be satisfactorily explained as a passive movement of water accompanying shifts of ions whose distribution in tissues is dependent upon a source of metabolic energy.

In the absence of a rigid cell membrane a water pump would be necessary to maintain within cells a total solute concentration of two to three times the concentration of the medium. However, such a high intracellular concentration should result in the entry of a fluid hypotonic to the medium in order that the water activity may become uniform throughout the tissue fluid when energy metabolism is inhibited. The fact that the fluid which entered the tissues during swelling was not hypotonic to the medium would seem to exclude the presence of such high intracellular concentrations. Although the presence of small differences in concentration cannot be excluded by the present study, the results are most simply explained on the assumption that osmotic equality exists between intra- and extra-cellular fluids of metabolizing tissues (or more correctly is higher within the cells only by the small pressure exerted by the membrane tension).

SUMMARY

1. The swelling of tissues *in vitro* which occurs when energy metabolism is disturbed was studied. Changes in water, sodium, chloride and potassium contents of slices of guinea-pig kidney cortex, rat liver and rat cerebral cortex were determined.

2. The fluid entering the tissues in the process of swelling was not water alone, as some previous workers have tacitly assumed, but a solution approximately isotonic with the medium. The quantity of sodium entering the tissue was accounted for by that which exchanged on an ionic basis with intracellular potassium plus that which entered together with chloride.

3. This finding invalidates the argument that tissue swelling is a movement of water alone attracted by a high intracellular osmotic pressure.

4. The dependence of fluid exchange of tissues on tissue metabolism can be explained by the requirement of metabolic energy for the maintenance of ionic gradients in tissues. Active extrusion of sodium from cells maintains a higher extracellular than intracellular concentration of this ion, which counterbalances the osmotic effect of intracellular non-diffusible colloid, thus preserving the normal cellular volume. Accumulation of sodium in cells when their metabolism is inhibited has as a consequence an increase of tissue chloride. The temporary rise in osmotic pressure within cells produced by such entry of ions of the medium manifests itself as an increase in tissue hydration. Re-establishing metabolic activity in the tissue will result in extrusion of sodium and chloride with reduction in tissue hydration.

5. The prevention of tissue swelling in hypertonic saline solutions, even when tissue metabolism is depressed, may also be explained on this basis.

6. The results are most simply explained on the assumption that osmotic equality exists between intra- and extra-cellular fluids of metabolizing tissues.

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APPENDIX

I. *Derivation of ΔP for the simplified system illustrated in Fig.* 1

Let ΔP equal the osmotic pressure difference between cell and medium. Then (Hitchcock, 1946) for the simple system illustrated in Fig. 1,

$$\Delta P = \boldsymbol{RT}\,(\Sigma \text{ intracellular ions} - \Sigma \text{ extracellular ions})$$

when molal concentrations of ions are summed and unit osmotic activity coefficients are assumed for each ion species.

With Na excluded from the cell,

$$\Delta P_1 = \boldsymbol{RT}\big[([K^+]_i + [Cl^-]_i + [A^{n-}]) - ([Na^+]_o + [K^+]_o + [Cl^-]_o)\big].$$

Substituting values for $[K^+]_i$ and $[Cl^-]_i$ obtained from equations (2) and (3) of the text, this expression becomes

$$\Delta P_1 = \boldsymbol{RT}\big[[A^{n-}] + \sqrt{\{n^2[A^{n-}]^2 + 4([K^+]_o\,[Cl^-]_o)\}} - 2[Cl^-]_o\big]. \quad (7)$$

With Na^+ freely entering the cell:

$$\Delta P_2 = RT[([K^+]_i + [Na^+]_i + [Cl^-]_i + [A^{n-}]) - ([Na^+]_o + [K^+]_o + [Cl^-]_o)].$$

Substituting values for $[K^+]_i + [Na^+]_i$ and for $[Cl^-]_i$ obtained from equations (5) and (6) of the text, this becomes

$$\Delta P_2 = RT[[A^{n-}] + \sqrt{\{n^2[A^{n-}]^2 + 4([K^+]_o [Cl^-]_o + [Na^+]_o [Cl^-]_o)\}} - 2[Cl^-]_o], \quad (8)$$

in which ΔP is expressed as a function of concentration and charge of intracellular non-diffusible anion, $[A^{n-}]$, and the concentrations of ions in the medium.

II. *Effect on ΔP_2 of varying the solute concentration in the medium when Na^+ is free to enter the cell*

Again, as $[Na^+]_o + [K^+]_o = [Cl^-]_o$, the total ionic concentration of the medium may be represented by $2[Cl^-]_o$ and equation (8) rewritten as

$$\frac{\Delta P_2}{RT} - [A^{n-}] = \sqrt{\{n^2[A^{n-}]^2 + 4[Cl^-]_o^2\}} - 2[Cl^-]_o. \quad (9)$$

As $[A^{n-}]$ is assumed to remain constant the effect of altering the medium concentration, $2[Cl^-]_o$, on the right-hand portion of equation (9) will be considered.

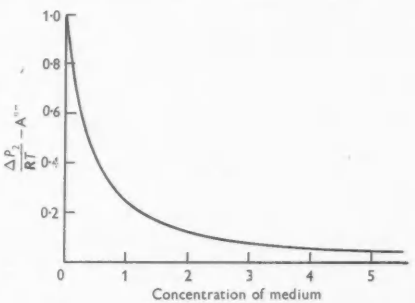

Fig. 2. Calculated values for the function $\Delta P_2/RT - [A^{n-}]$ from equation (9) of Appendix II which show the expected decrease of ΔP_2 (the difference in osmotic pressure between cell and medium when metabolism is inhibited) with increase of concentration of the medium. Units on both abscissa and ordinate are expressed as factors of $n[A^{n-}]$.

If $2[Cl^-]_o$ is much smaller than $n[A^{n-}]$ the right-hand expression has a maximum value of $n[A^{n-}]$. If $2[Cl^-]_o$ is much larger than $n[A^{n-}]$ the right-hand expression has a minimum value of 0. With $n[A^{n-}]$ kept constant this latter state can be approximated by increasing the concentration of medium, $2[Cl^-]_o$, and would have the effect of reducing the value of ΔP_2 to a value as low as any value for ΔP_1 that might exist. Fig. 2 shows a graph of calculated changes in $\Delta P_2/RT - [A^{n-}]$ with increase of medium concentration. Though the present results are inadequate to permit an accurate assessment of an actual value for $n[A^{n-}]$ it is likely that changes in the curve between concentrations of the medium of approximately 1 to 4 in Fig. 2 are pertinent to the present discussion.

III. *Decrease in sum of intracellular cation concentrations subsequent to entry of chloride into cells*

Referring again to the simplified model in Fig. 1 and letting subscripts 1 and 2 denote the intracellular ions before and after entry of Na^+ into the cell, we have

$$\Delta P_1 = RT[[K^+]_1 + [Cl^-]_1 + [A^{n-}]_1 - ([Na^+]_o + [Cl^-]_o + [K^+]_o)],$$

$$\Delta P_2 = RT[[K^+]_2 + [Na^+]_2 + [Cl^-]_2 + [A^{n-}]_2 - ([Na^+]_o + [Cl^-]_o + [K^+]_o)],$$

in which all ion symbols refer to concentrations.

Substituting for $[Cl^-]_1$ its value from equation (2) of text; ΔP_1 may be rewritten

$$\frac{\Delta P_1}{RT} = 2[K^+]_1 - n[A^{n-}]_1 + [A^{n-}]_1 - ([Na^+]_o + [Cl^-]_o + [K^+]_o). \quad (a)$$

Similarly, using equation (5) of the text, the expression for ΔP_2 becomes,

$$\frac{\Delta P_2}{RT} = 2[K^+]_2 + 2[Na^+]_2 - n[A^{n-}]_2 + [A^{n-}]_2 - ([Na^+]_o + [Cl^-]_o + [K^+]_o). \quad (b)$$

Subtracting (a) from (b)

$$(2[K^+]_2 + 2[Na^+]_2 - (n-1)[A^{n-}]_2) - (2[K^+]_1 - (n-1)[A^{n-}]) = \frac{\Delta P_2 - \Delta P_1}{RT}. \quad (c)$$

If the difference in osmotic pressures $\Delta P_2 - \Delta P_1$ is minimized by swelling of the cells, the right-hand expression in (c) approaches a limiting value of zero and (c) may be approximated by

$$2[K^+]_1 - (n-1)[A^{n-}]_1 = 2[K^+]_2 + 2[Na^+]_2 - (n-1)[A^{n-}]_2. \quad (d)$$

But the increase of fluid in the cells associated with tissue swelling will reduce the concentration of the fixed quantity of intracellular polyvalent anion so that

$$(n-1)[A^{n-}]_1 > (n-1)[A^{n-}]_2.$$

The effect of this inequality on equation (d) is to make

$$[K^+]_1 > [K^+]_2 + [Na^+]_2,$$

or the sum of the concentrations of intracellular cations decreases subsequent to entry of chloride into the cells.

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Ascorbic Acid and Glutathione as Respiratory Carriers in the Respiration of Pea Seedlings

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Szent-Györgyi (1931), from a knowledge of the behaviour of reduced glutathione (GSH) and ascorbic acid (AA), first postulated a scheme whereby these substances might act as respiratory carriers. The subsequent discovery of the enzymes dehydroascorbic acid reductase (Crook & Hopkins, 1938; Crook, 1941) and glutathione reductase (Mapson & Goddard, 1951; Conn & Vennesland, 1951) made it possible to visualize a system whereby hydrogen may be transferred from substrates such as *iso*citrate and malate to molecular oxygen. Mapson & Goddard (1951), in fact, were able to demonstrate the reduction of dehydroascorbic acid (DHA) as a result of hydrogen transfer from either malate or *iso*citrate via triphosphopyridine nucleotide (TPN) and GSH. Such a system might be expected to consume oxygen if, in addition to these enzymes, a terminal oxidase capable of oxidizing ascorbic acid was present. The reactions concerned may be formulated as follows:

$$SH_2 + TPN^+ \xrightarrow{\text{dehydrogenases (specific for TPN)}} TPNH + H^+ + S$$

$$TPNH + H^+ + GSSG \xrightarrow{\text{glutathione reductase}} TPN^+ + 2GSH,$$

$$2GSH + DHA \xrightarrow{\text{dehydroascorbic reductase}} AA + GSSG,$$

$$AA + \tfrac{1}{2}O_2 \xrightarrow{\text{enzymes oxidizing ascorbic acid}} DHA + H_2O,$$

where SH_2 represents an oxidizable substrate.

The work described here aimed to demonstrate the presence of such a system in extracts *in vitro*, and to evaluate its importance in the respiration of the tissue *in vivo*.

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EXPERIMENTAL

Preparation of materials and reagents

Pea seedlings. Pea seeds, var. Kelvedon Wonder, were sterilized by soaking in 1% (w/v) $HgCl_2$ solution for 1 min., washed thoroughly in running tap water, soaked for 24 hr. in tap water and then germinated for the appropriate period at 20°. In the designation of age of seedling the soaking period is included as the first day of germination.

Triphosphopyridine nucleotide (TPN). The method of preparation of Warburg, Christian & Griese (1935), as modified by Haas (unpublished), was normally used. In some cases the method of LePage & Mueller (1949) was employed. The purity was determined according to the method used by LePage & Mueller (1949), except that the enzyme *iso*citric dehydrogenase was used instead of glucose 6-phosphate dehydrogenase.

Dehydroascorbic acid (DHA). This was prepared by dissolving the equivalent amount of ascorbic acid in distilled water and adding bromine drop by drop until a faint orange colour of bromine appeared. The excess bromine was removed by aeration and the solution was brought to pH 6·5 by the addition of a solution of 20% (w/v) Na_2HPO_4.

Glutathione. The reduced form (GSH) was obtained from British Drug Houses Ltd. Oxidized glutathione (GSSG) was prepared by dissolving the appropriate amount of the reduced form in water, adding a drop of starch indicator and titrating with an iodine solution until a very faint blue colour appeared. This solution was freshly prepared before each experiment.

Sodium isocitrate. This was prepared from DL-*iso*citric acid lactone, obtained from H.M. Chemical Co. Ltd., California, U.S.A. The lactone solution was heated at 100° with the equivalent amount of NaOH for 20 min. This solution was stored at −20°.

Sodium malate. This was prepared from malic acid, purchased from Hopkin & Williams Ltd.

Methods

Oxygen uptake. Oxygen consumption was measured in Warburg manometers at 25° in air. The fluid vol. was 3 ml., including 0·2 ml. of 20% KOH in the centre well to absorb CO_2. The flasks were equilibrated by shaking for at least 10 min. The reaction was started by tipping the substrate from the side arm.

Fractionation of cell constituents. As the mitochondria are well known to be sensitive to high temperature, all apparatus and solutions were cooled to 1° before use, and the extracts obtained kept at this temperature until the start of the experiments. Germinated pea seeds (60 g.) were ground in a mortar to a fine paste with 80 ml. of 0·1M phosphate buffer, pH 7·4, containing 0·4M sucrose and 0·004M-$MgSO_4$. The extract was then centrifuged at 500 *g* for 5 min. The residue, which contained most of the starch and cell debris, was discarded and the supernatant, referred to in the text as the 'suspension', was recentrifuged at 20 000 *g* for 20 min. at −3°. Under these conditions, the cytoplasmic particles (mitochondria) are sedimental, and the supernatant represents the soluble part of the cytoplasm. The mitochondria were washed by suspending in 60 ml. of the same sucrose buffer solution and recentrifuged at 20 000 *g* for 20 min. They were then taken up in a volume of sucrose buffer equal to that of the original extract.

The respiration of these cell fractions was determined as quickly as possible after their preparation, usually within 1 hr. of starting extraction, and the same applied to the extracts used for studying the rate of reduction of DHA.

Determination of ascorbic acid. Direct visual titration against 2:6-dichlorophenolindophenol was used according to the method of Harris & Olliver (1942).

Determination of reduced glutathione. GSH was determined by titration against potassium acid iodate, using the method of Fujita & Numata (1938).

Determination of enzyme activity. Ascorbic oxidase activity was measured by determining the catalytic effect of the extract on the oxidation of ascorbic acid at 25°. The rate of oxidation of the substrate was followed by removal of samples into metaphosphoric acid, and determining the unoxidized ascorbic acid by titration against indophenol dye.

The estimation of the activity of dehydroascorbic acid reductase was carried out by determining the rate of formation of ascorbic acid on adding GSH (3 mg./ml.) and DHA (1 mg./ml.) to the extract under anaerobic conditions.

Glutathione reductase activity was measured by determining the rate of formation of GSH on the addition of malate, TPN, $MnCl_2$ and GSSG, under anaerobic conditions according to the method described by Mapson (1953).

Determination of CO_2 production by intact seeds. The CO_2 produced by 10–15 g. of seedlings was absorbed in 0·2N-$Ba(OH)_2$ solution, the CO_2 being determined by back titration with standard HCl.

RESULTS

If ascorbic acid and glutathione act as respiratory carriers in a scheme similar to that suggested in the introduction, it should be possible to demonstrate the consumption of oxygen by extracts in which all the enzymes of this system are present. One tissue in which all these enzymes could be demonstrated was pea seeds which had been allowed to germinate for 5 days at 20° and which were extracted as follows: Pea seedlings (50 g.) were ground with 50 ml. of 0·1M phosphate buffer, pH 6·8, and left at room temperature for 15 min. with occasional stirring. The mixture was then centrifuged at 500 *g* for 5 min., and the supernatant was recentrifuged at 20 000 *g* for 30 min. The clear final supernatant was dialysed at 1° for 72 hr. against 5 l. of 0·1M phosphate buffer, pH 6·8, which was changed every 24 hr. It was necessary to prolong the dialysis for this period in order to remove completely substrates and/or coenzymes to reduce oxygen uptake to zero. Air was bubbled through the outside solution to facilitate dialysis.

In this enzyme preparation the presence of malic enzyme, GSSG reductase, DHA reductase together with ascorbic acid oxidase was established in separate experiments. Further, DHA, when added to the preparation, was reduced under anaerobic conditions.

As shown in Fig. 1, the enzyme preparation without the addition of any substrate or coenzyme showed no oxygen uptake. Five different experiments were carried out in order to reveal the presence of a GSH–AA hydrogen-transfer system. The substrates and coenzymes added to the enzyme extract which contained 4×10^{-3}M-Mn^{2+} or -Mg^{2+} were as follows: (1) AA and GSH; (2) malate and TPN; (3) malate, TPN and AA; (4) malate, TPN and GSH; and (5) malate, TPN, GSH and AA.

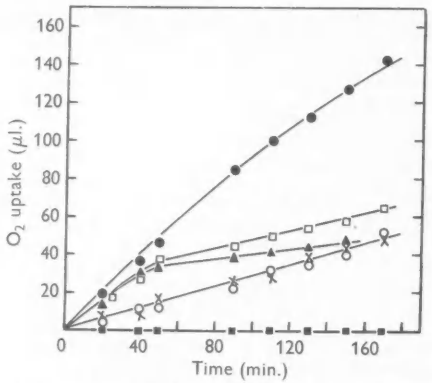

Fig. 1. Oxygen uptake due to a GSH–ascorbic acid enzyme system in dialysed extracts from 5-day germinated peas. Each flask contained 2 ml. of enzyme extract in a volume of 2·8 ml. ■, Dialysed extract; ○, extract + malate (2 mg./ml.) + TPN (30 μg./ml.); ×, extract + malate + TPN + GSH (50 μg./ml.); □, extract + malate + TPN + AA (50 μg./ml.); ▲, extract + GSH + AA; ●, extract + malate + TPN + AA + GSH.

Expt. 1 showed that on the addition of ascorbic acid and GSH an initially rapid but brief oxygen uptake was observed, which was equivalent to the amount required to oxidize both the ascorbic acid and GSH added. It appears, therefore, that there was no regeneration of ascorbic acid or GSH under these conditions.

In Expt. 2, on the addition to the enzyme preparation of malate and TPN, a low but continuous oxygen uptake was observed which may have been due to hydrogen transfer from TPNH via flavoprotein enzymes to molecular oxygen, for it was not abolished by 10^{-3}M cyanide. In Expt. 3, on the further addition of ascorbic acid, oxygen consumption was increased initially only by the amount required to oxidize the ascorbic acid. In Expt. 4, when GSH was added the rate of oxygen uptake was no greater than that observed in Expt. 2, indicating that in the absence of ascorbic acid or its oxidized product no oxidation of GSH occurred. Only in Expt. 5, where all components of the system were present, was there a rapid and continuous oxygen uptake. After 3 hr. the experiments were discontinued and the contents of the Warburg flasks analysed for ascorbic acid; only in the flask containing the complete system was any ascorbic acid detected.

Essentially similar results were obtained when DHA and GSSG were substituted for ascorbic acid and GSH, save that the small initial oxygen uptake due to the oxidation of the reduced forms was eliminated.

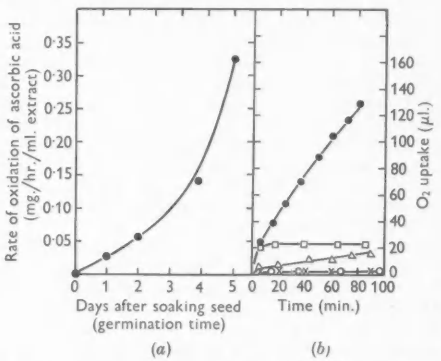

Fig. 2. Development of ascorbic acid oxidase activity during germination, and the effect of adding this enzyme on the oxygen consumption of extracts prepared from pea seeds in the early stages of germination. Concentrations of TPN, malate, GSH and ascorbic acid are as in Fig. 1. (*a*) Ascorbic acid oxidase activity of pea-seed extracts at different stages of germination. (*b*) O_2 uptake of dialysed extracts prepared from pea seeds after soaking for 24 hr. ×, Dialysed extract; ○, extract + AA oxidase preparation; □, extract + AA oxidase preparation + AA + GSH; △, extract + TPN + malate + GSH + AA; ●, extract + AA oxidase + TPN + malate + GSH + AA.

Attempts to demonstrate the presence of such a system in dialysed extracts obtained from pea seeds in the very early stages of germination (soaked for 1 day) were unsuccessful. This was apparently due to the absence of an enzyme catalysing the oxidation of ascorbic acid, for the rate of oxidation of this substance when added to extracts prepared from such seeds was low. This is shown in Fig. 2 (*a*), where the development of ascorbic acid oxidase as the seed germinates is illustrated. The assumption that the failure to demonstrate the GSH–ascorbic acid system was due to lack of a suitable terminal oxidase was found to be correct by showing (1) that all the other enzymes of the system were present, (2) that on addition of DHA, GSSG, TPN and malate, ascorbic acid was formed, and (3) that the addition of an

ascorbic acid oxidase preparation from cucumber (Crook, 1941) led to an immediate consumption of oxygen, which, as before, was shown to be due to the participation of GSH and ascorbic acid (Fig. 2*b*).

The consumption of oxygen, due to the operation of the GSH–ascorbic acid system, was also shown to occur in dialysed extracts prepared from peas germinated for longer periods (6–7 days). It was noteworthy, however, that as the germination of the seedlings progressed, the oxygen uptake stimulated by the addition of TPN and malate alone to the enzyme extract increased relatively to the oxygen uptake directly attributable to the GSH–ascorbic acid system. Results showing this are given in Table 1. It seems clear that as germination progresses the activity of enzymes capable of catalysing the direct oxidation of TPNH (for convenience collectively termed 'diaphorases') increases relatively to the activity of the enzymes associated with the reduction and oxidation of GSH and ascorbic acid.

Table 1. *Proportion of oxygen uptake of dialysed extract of peas attributable to 'diaphorase' enzymes and enzymes associated with GSH–ascorbic acid system*

Dialysed extract prepared from	Percentage of oxygen uptake due to: Diaphorase system	Percentage of oxygen uptake due to: GSH–ascorbic acid system
2-Day germinated peas + ascorbic oxidase	20	80
5-Day germinated peas	40	60
7-Day germinated peas	58	42

The fact that these enzymic reactions can be demonstrated in plant extracts *in vitro*, however, gives no indication to what extent the normal respiration of the tissue proceeds by this route *in vivo*.

Intracellular localization of enzymes oxidizing and reducing glutathione and ascorbic acid in pea seedlings

It was thought that a knowledge of the localization of the enzymes associated with the GSH–ascorbic acid system within the cell might be of interest in evaluating the importance of the system in respiratory processes. The activity of each of the enzymes specifically associated with this system (i.e. ascorbic acid oxidase, dehydroascorbic acid reductase and glutathione reductase) was accordingly determined in (*a*) the suspension (mitochondria + soluble part of the cytoplasm), (*b*) the mitochondria, and (*c*) the soluble part of the cytoplasm as obtained from pea seeds which had been germinating for 1, 5 or 7 days. The presence of all these enzymes could be demonstrated in the suspension prepared from such seeds. The activity in the suspension of dehydroascorbic acid reductase could be entirely attributed to its activity in the soluble part of the cytoplasm, which was confirmed by the observation that intact mitochondria prepared from seedlings after 1, 5 or 7 days germination failed to catalyse the reduction of DHA. Enzymes catalysing the reduction of GSSG or the oxidation of ascorbic acid were also mainly concentrated in the soluble part of the cytoplasm. An oxidase catalysing this oxidation, although absent from the mitochondria prepared from seedlings after 1-day germination, was present in these particles after 7 days germination. However, even in the latter case, it accounted for only 10% of the total oxidase activity of the suspension.

Our failure to demonstrate any appreciable ability of mitochondria to reduce DHA and GSSG may have been due to either the inaccessibility of added substrates to the enzymes present in the intact mitochondria, or to the absence of the enzymes from these cytoplasmic particles. It was essential, for our purpose, to decide which of these interpretations was correct. Accordingly, we attempted to disintegrate the mitochondria by methods which have been used by many workers to prepare soluble enzymes from cytoplasmic particles. Three methods were used: (1) treatment with cold acetone at $-20°$ (Hogeboom, Claude & Hotchkiss, 1946; Drysdale & Lardy, 1953), (2) addition of surface-active agents such as octan-2-ol (Huennekens & Green, 1950), and (3) homogenization in a Waring Blendor (Still & Kaplan, 1950).

The treatment with acetone was carried out as follows: Mitochondria were washed with sucrose buffer and suspended in a small volume of sucrose–phosphate buffer. Excess of cold acetone at $-20°$ was then added and the mixture was centrifuged. The acetone layer was decanted and a stream of nitrogen was passed into the centrifuge tube to remove the remaining acetone. The residue was then taken up in the appropriate volume of sucrose buffer and used for enzymic tests.

In the second method, 0·02 ml. of octan-2-ol was usually added to each ml. of mitochondrial preparation.

In the third method, homogenization was carried out in a micro-Waring Blendor for 6 min. at 1–5°.

It was first necessary to show that the enzymes themselves were not affected by any of these treatments. The enzymes in the soluble fraction of the cytoplasm were, therefore, subjected to each of these procedures and the activity was tested before

and after. The results showed that none of these treatments had any adverse effects on ascorbic acid oxidase or dehydroascorbic acid reductase. Glutathione reductase was not affected by either the octan-2-ol or acetone treatment, but there was a slight decrease (30%) in its activity after blending.

No evidence for the presence of an enzyme catalysing the reduction of DHA was obtained when mitochondria were subjected to these treatments, nor was the oxidase activity associated with intact mitochondria increased by any of them. It therefore seemed that the enzyme specifically involved in the reduction of DHA was associated with the soluble part of the cytoplasm and not with the mitochondria.

In contrast to the conclusions of Rall & Lehninger (1952) for liver and of Davies (1954) for peas, we have found glutathione reductase in pea mitochondria, but its activity was at the most only about one-fifth of its activity in the soluble fraction.

Relation between the oxygen consumption of pea suspensions and the respiration of intact pea seedlings

The fact that the enzymes associated with the reduction of DHA and GSSG are mainly located in the soluble part of the cytoplasm made it possible to determine more closely the part played by the system in total respiration. Thus electron transfer via the system could not be more than the total respiratory activity of the soluble part of the cytoplasm itself. If, therefore, the respiratory activities of this fraction and of the mitochondria could be separately estimated, the role played by the GSH–ascorbic acid system could be more closely defined.

It was first necessary to determine whether during extraction of the different cellular fractions the respiration of the tissue had been seriously affected, because unless the respiration of the freshly prepared suspension is of the same order as that of the intact tissue, the results may obviously rest on an erroneous basis. Secondly, the procedure used in the further separation of mitochondria and soluble fraction must be such that the sum of the respiration of the separated fractions is equal to that of the suspension. The following experiments were accordingly carried out to establish these points.

Pea seeds were germinated at 20°. After 5 days, 10–15 g. of seedlings were incubated at 25° and their CO_2 production was measured. At the same time a further sample was extracted as described earlier, and the oxygen uptake of the suspension determined in the Warburg apparatus. On the assumption that the R.Q. was 1·0, the respiration of the intact seedlings was expressed as μl. of oxygen/g. fresh weight of seedlings/hr., and the oxygen uptake of the suspension similarly. The results showed that the respiration of the suspension was of the order of 83% of that of the intact tissue. There are reasons, however, for believing that this estimate is low. In the preparation of suspensions from the seedlings, large amounts of starch and cell debris are discarded. Some of the mitochondria are, no doubt, lost with the cell debris or disintegrated during the preparatory procedures. There is no direct method of determining this loss, which Millerd (1953) thought might be as high as 50%. Some further experiments were, therefore, carried out in an attempt to allow for this. In these experiments the nitrogen content, as representing the total protein content, was taken as the basis for the calculation of the oxygen consumption. The results obtained (Table 2) show an even closer agreement between the oxygen consumption of the suspension and that calculated for the intact tissue.

In the preparation of these tissue suspensions, besides disruption of the cells some dilution of the constituents of the protoplasm was inevitable. We have tried to find out whether this dilution had affected the oxygen uptake. It was shown that a further twofold dilution of suspensions, prepared either from cotyledons or detached embryos, reduced the oxygen consumption strictly in accordance with the degree of dilution. There is

Table 2. *Comparison of the oxygen consumption of whole seedlings, cotyledons and detached embryos, with that of suspensions prepared from these tissues*

	Intact tissue		Suspension	Intact tissue		Suspension
Material	O_2 uptake calc. from CO_2 production (μl./g./hr.)	O_2 uptake measured directly (μl./g./hr.)	O_2 uptake equivalent to 1 g. of tissue (μl./g./hr.)	O_2 uptake calc. from CO_2 output (μl./mg. N/hr.)	O_2 uptake measured directly (μl./mg. N/hr.)	O_2 uptake equivalent to 1 g. of tissue (μl./mg. N/hr.)
Whole seedling (5 days)	242*	—	210	13·4	—	13·1
Cotyledon (5 days)	250*	321, 252	220	12·7	16·3 12·7	12·1
Detached embryo (5 days)	664†	695, 695	83	96·5	101, 101	15·3
Cotyledon (7 days)	—	300, 246	200	—	19·4, 15·8	13·8
Detached embryo (7 days)	—	684, 684	84	—	107, 107	17·9

* Mean of hourly readings over a 6 hr. period. † Mean of hourly readings over a 2 hr. period.

thus no evidence that the oxygen consumption of these suspensions was altered in other respects merely as a result of any dilution of the cell cytoplasm.

Cotyledons were detached from the rest of the embryo (plumule and radicle) and the experiments were repeated with both these tissues. In addition, the oxygen uptakes of the intact cotyledon and of the detached embryo were directly determined in the Warburg apparatus; there was good agreement, considering the size of samples used (1 g.), between the oxygen uptake thus measured, and the oxygen consumption as calculated from the CO_2 production of 10–15 g. of these tissues. A comparison of the respiration of the intact tissues with the oxygen consumption of the corresponding suspensions revealed an important difference between the cotyledon and detached embryo. With the former, the oxygen consumption of the intact tissue, whether calculated from the CO_2 production or by direct measurement, agreed reasonably well with that of the suspension, but with the latter the oxygen consumption of the suspension was only about one-tenth of that of the intact tissue (Table 2). Clearly, in the latter case the oxygen consumption had been altered so much during extraction that we felt it impossible from results with such extracts to draw any conclusions about the part played by the GSH–ascorbic acid enzyme system in the respiratory activity. Nevertheless, as shown later, we have analysed the respiration of suspensions from the detached embryo in the same way as that used for similar extracts from the cotyledon, in the hope that the results might be of some interest. The good agreement reported above for the oxygen consumption of the intact whole seedling with that of suspensions was obviously due to the fact that the cotyledon comprised 87% of the weight of the seedling at this stage of germination (5 days).

Oxygen consumption of the mitochondria and soluble fraction of the cytoplasm

Having established for the cotyledon that the procedure used to extract these cellular components did not appear to alter significantly their respiratory activity, we proceeded to separate mitochondria from the soluble fraction of the suspension and to determine their separate oxygen consumptions. When this was carried out on peas germinated for 5 days it was found that the sum of the oxygen consumption of washed mitochondria and of the soluble fraction amounted to only 70% of the total oxygen consumption of the suspension. This discrepancy appears to be due to an artificial depression of the respiration of the mitochondria due to a lack of substrates and/or coenzymes. Millerd (1953) determined the oxygen uptake of washed mitochondria after adding succinate in arbitrary amounts, and this was taken to indicate the respiration of these cytoplasmic particles under physiological conditions. A better evaluation of the respiration of mitochondria under physiological conditions would appear to be obtained if the substrates, as distinct from the enzymes, in the soluble part of the cytoplasm were added to these cytoplasmic particles. In this case we should be determining the oxygen uptake in the presence of the substrates which are normally in contact with the mitochondria in the living cell. This was achieved by measuring the oxygen uptake of washed mitochondria in the presence of the soluble part of the cytoplasm, in which the enzymes had previously been inactivated by heating for 2 min. at 100°. When this was done the oxygen consumption of the mitochondria was increased to a value which was 2–3 times that of the washed mitochondria alone (Fig. 3).

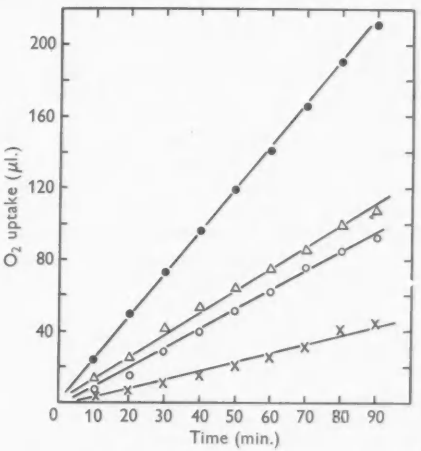

Fig. 3. Oxygen uptake of cellular fractions from 5-day germinated seeds. ●, Mitochondria + soluble part of cytoplasm (suspension); ○, mitochondria + heated soluble part of cytoplasm; ×, washed mitochondria in equivalent concentration; △, equivalent amount of soluble part of cytoplasm.

This technique was applied to pea seeds which had been germinated for 2, 5 and 7 days and the oxygen consumption of the suspension, mitochondria and soluble fraction determined. The oxygen uptake of the two latter fractions on summation accounted for 90, 97 and 99% respectively of the total respiration of the suspension prepared from the same seedlings.

This method of estimating the oxygen consumption of the mitochondria and of the soluble

fraction was further checked by determining the oxygen consumption of the suspension and soluble fractions directly and obtaining that of the mitochondria by difference; results by either method agreed extremely well.

The distribution of the oxygen uptake between these cell fractions prepared from the cotyledon was found to be almost equally divided between the mitochondria and soluble fraction. It follows from this, and from the distribution of the enzymes associated with the GSH–ascorbic acid system, that the latter cannot account for, at the most, more than half of the respiration of the cotyledons.

In similar experiments with suspensions from detached embryos the oxygen consumption of the soluble fraction remained fairly constant over the period studied (5–9 days), but that of the mitochondria progressively increased. At 5 days 70% of the total respiration of the extract resided in the soluble fraction, but this decreased to a value of 50% over the next 4 days.

Inhibitor studies

Having shown that the participation of the GSH–ascorbic acid system in the transfer of electrons cannot be greater than that represented by the oxygen uptake of the soluble part of the cytoplasm, we used enzyme inhibitors in an attempt to determine the proportion of the oxygen consumption of this cell fraction which could properly be ascribed to this enzyme system. Since we found in our extracts no enzymes capable of oxidizing GSH in the absence of dehydroascorbic acid, the whole of the electron transfer must proceed via ascorbic acid. It followed that the easiest way of blocking the system was to prevent the functioning of those enzymes which oxidize ascorbic acid. In peas the oxidases most likely to be involved are ascorbic oxidase and cytochrome oxidase. Fortunately the latter enzyme appears to be exclusively confined to the mitochondria; certainly, we have found no evidence for its presence in the soluble part of the cytoplasm. We could therefore employ reagents such as cyanide or sodium diethyldithiocarbamate as inhibitors, without fear that the inhibition observed was due to interference with other respiratory pathways involving cytochrome oxidase.

If it could be shown that the maximum degree to which the oxygen consumption of the soluble fraction is depressed is the same for different inhibitors all capable of inactivating copper proteinates we should have suggestive evidence that the inhibition observed was due to the inactivation of enzymes capable of oxidizing ascorbic acid, and therefore that the fall in oxygen uptake was due to inactivation of an ascorbic acid system.

It was first essential to determine which of the known inhibitors of copper proteinates would prevent the oxidation of ascorbic acid in our extracts. Of those tested cyanide, diethyldithiocarbamate and 8-hydroxyquinoline all inhibited at millimolar concentration in extracts prepared from seedlings at all stages of germination studied. The inhibition with cyanide and diethyldithiocarbamate was complete at this concentration but with 8-hydroxyquinoline the percentage inhibition was somewhat less (88%).

The oxygen consumption of the soluble fraction of the cytoplasm prepared from pea seedlings after 2, 5 and 7 days germination was accordingly determined in the presence of cyanide and of diethyldithiocarbamate. In all cases the maximum percentage inhibition observed with both compounds was, within experimental error, the same and was obtained at concentrations above 5×10^{-4}M (Table 3*a*).

The fact that the oxygen consumption of all these extracts was affected to a similar degree by cyanide and diethyldithiocarbamate makes it probable that much of the respiration is normally proceeding through oxidases containing copper as the prosthetic group, and of these the most likely to be involved are those concerned with the oxidation of ascorbic acid.

Reduction of DHA by soluble fraction of cytoplasm

Although such results are most simply explained on the assumption that cyanide and diethyldithiocarbamate have prevented the functioning of a respiratory chain involving ascorbic acid, it cannot be accepted, on this evidence alone, that the GSH–ascorbic acid system is responsible for the whole of the cyanide-sensitive respiration of the soluble part of the cytoplasm.

The work of Matthews (1951), Nason, Wosilait & Terrell (1954) and Kern & Racker (1954) has shown the existence of an enzyme in peas catalysing electron transfer from DPNH to an acceptor which appears to be an intermediate oxidation product between ascorbic and dehydroascorbic acid; in these reactions dehydroascorbic acid cannot act as an acceptor. It is thus possible that some of the cyanide-sensitive respiration of the soluble fraction is due to this system, since, like the GSH–ascorbic acid system it would not function in the absence of enzymes oxidizing ascorbic acid.

In an attempt to obtain further evidence, we have estimated the rate of hydrogen transfer through the GSH–ascorbic acid system by measuring the rate of reduction of DHA when this substance was added to the soluble enzyme fraction. This may be most simply accomplished by measuring the rate of reduction in the absence of oxygen. Such a procedure, while preventing the

oxidation of ascorbic acid by ascorbic acid oxidase, also measures the full reducing potential of the extract towards DHA, but at the same time may give erroneous results since the action of diaphorase enzymes oxidizing TPNH and therefore normally acting competitively with glutathione reductase would be eliminated by exclusion of oxygen. A better, though by no means ideal, evaluation of the rate of reduction can, however, be obtained by using cyanide under aerobic conditions, in which circumstances oxidation of ascorbic acid is prevented, without the total exclusion of other enzyme systems. The presence in wheat of TPNH-oxidizing enzymes which are partially inhibited by 10^{-3}M cyanide has been shown by Conn, Kraemer, Liu & Vennesland (1952) and by Humphreys (1955).

Analysis of the soluble fraction of the cytoplasm prepared from the whole seedling

We have used these techniques in an attempt to evaluate the importance of the GSH–ascorbic acid system in the respiration of the developing seedling. In the first instance this analytical approach was applied to the soluble fraction of the cytoplasm obtained from the whole embryo (cotyledon, plumule and radicle) with results (Table 3*b*) which showed that the oxygen uptake as computed from the rate of reduction of DHA, accounted for the major proportion (70%) of the respiration inhibited by cyanide and diethyldithiocarbamate. In the earlier stages of germination the rate of reduction of DHA was identical under anaerobic or aerobic conditions where cyanide was present to

Table 3. *Effect of* 10^{-3}M *cyanide and diethyldithiocarbamate on the respiration of the soluble fraction of the cytoplasm prepared from suspensions of whole seedlings and the rate of reduction of DHA by the same fraction*

(*a*)

	O_2 uptake of soluble part of cytoplasm (μl./g. fresh wt./hr.)						
Material	Total	Cyanide-insensitive	Cyanide-sensitive	Inhibition of respiration by cyanide (%)	Diethyldithiocarbamate-insensitive	Diethyldithiocarbamate-sensitive	Inhibition of respiration due to diethyldithiocarbamate (%)
2-Day seedling	10	0	8	80	0	8	80
2-Day seedling (with added AA oxidase)	53	4	49	92	6	47	89
5-Day seedling	78	24	54	69	26	52	67
7-Day seedling	95	56	39	41	60	35	37

(*b*)

	Rate of reduction of DHA by the soluble part of cytoplasm expressed as equivalent of μl. O_2/g. fresh wt./hr.			Respiration as measured by rate of reduction of DHA in air with KCN	
Material	In N_2	In air with 10^{-3}M-KCN	Rate in air as % of that in N_2	Percentage of cyanide-sensitive respiration of soluble fraction	Percentage of total respiration of soluble fraction
2-Day seedling	34	34	100	79*	64*
5-Day seedling	39	37	96	69	47
7-Day seedling	40	28	70	72	30

* Calculated values obtained from reduction of DHA by extract, with respiration of extract with added ascorbic acid oxidase.

It was further of importance to ascertain that the rate of reduction was independent of the concentration of DHA over the range tested (0·03–0·40 mg./ml. reaction mixture), which suggests that in these extracts the rate-limiting step in the transfer of hydrogen through the GSH–ascorbic acid system is the rate of reduction of GSSG rather than that of DHA.

prevent oxidation of the ascorbic acid formed; only with older seedlings was there any marked influence of oxygen on this reaction. The results included in Table 3*b* show that the rate of reduction of DHA with 2-day germinated peas was as great as that observed with older peas, suggesting that the low oxygen consumption of the soluble fraction prepared from the former peas was not

due to any deficiency of substrates, enzymes or coenzymes associated with the reduction of GSSG or DHA but rather to the lack of enzymes capable of oxidizing ascorbic acid. This was subsequently confirmed by the observation that the respiration could be greatly increased by the addition of ascorbic acid oxidase (Table 3*a*).

Activity of the GSH–ascorbic acid system in cotyledons and detached embryos

The above results gave only a very approximate overall picture of the role played by this enzyme system in the developing seedling, and no attempt was made to determine whether the system was operating in the respiration of the cotyledon as distinct from the detached embryo (plumule + radicle detached from the cotyledon). Unless the enzymes associated with oxidation and reduction of GSH and ascorbic acid, and other enzymes which may act competitively with this system, are equally distributed throughout these tissues, the results obtained from an extract of the mixed tissue may be misleading.

One difference between the cotyledon and the detached embryo was the very uneven distribution of ascorbic acid oxidase. We have already shown the development of this enzyme in extracts prepared from whole seedlings, and we were therefore surprised to find that although this enzyme did increase in the cotyledon during germination its activity in this tissue was only about 5% of its activity in extracts from the detached embryo. Whilst the activity of the oxidase in the soluble fraction of the cytoplasm prepared from cotyledons was only just adequate to account for the cyanide-sensitive respiration of this fraction, its activity in similar extracts from the detached embryo was some fifty times greater than that necessary to account for the cyanide-sensitive respiration observed. Assuming the oxygen consumption of these extracts, though reduced in magnitude, to be representative of the oxygen consumption of the intact tissue, the cyanide-sensitive component would be about ten times that observed, in which case the activity of the oxidase would be only five times that necessary for an enzyme acting as a terminal oxidase. Moreover, it has to be remembered that the assay of the ascorbic acid oxidase activity is necessarily carried out on dialysed extracts, in order to eliminate the reducing systems; hence the observed enzyme activities, especially the very high activity of extracts from detached embryo, may be very different from their real activity *in vivo*. It would seem worth while to pursue this point further in subsequent work.

We found that the other enzymes associated with the GSH–ascorbic acid system were present in extracts of the soluble fraction of the cytoplasm prepared from either cotyledons or detached embryos, as shown by the ability of such extracts to reduce DHA under anaerobic conditions. Within a period of 5 days after the start of germination there was no difference in the rate at which DHA was reduced between comparable extracts prepared from either the cotyledons or detached embryos but, whilst the activity of the former remained constant throughout the period of germination studied (9 days) the activity of the detached embryo tissue decreased by about half in the later stages (Table 4, col. 6). A further difference was that, whilst oxygen did not affect the rate of reduction of DHA in extracts from cotyledons containing cyanide, it reduced the rate of the reaction in extracts from the detached embryo, indicating that the cyanide-insensitive diaphorase enzymes oxidizing TPNH, already referred to, were absent from the cotyledons but present in the detached embryo. It was therefore to be expected that cyanide would inhibit the oxygen uptake of the soluble fraction of the cytoplasm from the cotyledons to a greater extent than from detached embryos, and this was found to be so (Table 4, col. 5). With 5- to 9-day-old cotyledons 70% of the total oxygen uptake of the soluble fraction was cyanide-sensitive and, from the rate of reduction of DHA in air with cyanide (Table 4. col. 7), 60% of this could be ascribed to electron transfer via GSH and ascorbic acid (Table 4, col. 9), which means that a little less than half of the total oxygen consumption of the soluble fraction of the cytoplasm may pass over this route (Table 4, col. 10).

The rate of reduction of DHA, by extracts from 2-day-old cotyledons, was almost as great as with extracts from longer-germinated peas (Table 4, cols. 6 and 7) and was equivalent to an electron transfer about three times the total oxygen consumption of the soluble fraction (Table 4, col. 2). As already pointed out, the low oxygen consumption of these extracts from 2-day-old cotyledons is due to the absence of a terminal oxidase. If, however, the oxygen consumption of such extracts is measured after the addition of ascorbic acid oxidase (Table 3*a*, col. 2), we may calculate, from such data, the percentage of the cyanide-sensitive oxygen consumption which may be ascribed to electron transfer via DHA. The result shows (Table 4, col. 9) that this percentage in the presence of an oxidase would be similar to that found with cotyledons from peas in a more advanced state of germination.

With suspensions from the detached embryo about one-third of the oxygen consumption of the soluble fraction was inhibited by cyanide (Table 4, col. 5), and from the corresponding data on the reduction of DHA in air with cyanide (Table 4, col. 7) nearly all of this could be accounted for by

an electron transfer over the GSH–ascorbic acid system (Table 4, col. 9).

With extracts from the whole embryo the apparent decrease in cyanide-sensitivity of the oxygen uptake of the soluble fraction (Table 3*a*, col. 5), and the increasing effect of oxygen on the reduction of DHA (Table 3*b*, col. 4), which were observed during germination, were evidently due to the increasing weight of the plumule + radicle relative to that of the cotyledons.

spondence between levels of respiration of intact tissue and extract being merely fortuitous, but this seems unlikely because experience shows that where serious disorganization of the protoplasm has occurred, respiratory activity is usually grossly affected, as was indeed the case with suspensions from the detached embryos. There is the further complication, which may ultimately explain the difference between the cotyledon and detached embryo, namely that with vacuolated tissues the

Table 4. *Effect of cyanide on the oxygen consumption of the soluble fraction of the cytoplasm prepared from suspensions of cotyledons and of detached embryos, and on the rate of reduction of DHA by the same fractions*

Material	O_2 uptake of soluble fraction (μl./g. fresh wt./hr.)				Rate of reduction of DHA (expressed as equivalent of μl. O_2/g. fresh wt./hr.)		Rate in air with KCN as % of that in N_2	Respiration as measured by rate of reduction of DHA in air with cyanide	
	Total	Cyanide-insensitive	Cyanide-sensitive	Inhibition by cyanide (%)	In N_2	In air with KCN		Percentage of cyanide-sensitive respiration of soluble fraction	Percentage of total respiration of soluble fraction
Cotyledons; 10^{-3} M cyanide									
2-Day cotyledon	11	0	11	100	30	30	100	61*	57*
5-Day cotyledon	85	30	55	65	39	39	100	71	46
7-Day cotyledon	93	27	65	71	39	39	100	59	42
9-Day cotyledon	85	25	60	70	36	36	100	60	42
Detached embryos (plumule + radicle); 2×10^{-3} M cyanide									
5-Day detached embryo	64	43	21	33	39	25	64	118	39
7-Day detached embryo	45	21	23	51	30	23	75	100	51
9-Day detached embryo	47	31	16	34	19	13	68	81	28

* Calculated values obtained from rate of reduction of DHA by extract and oxygen consumption of the extract with added ascorbic acid oxidase.

DISCUSSION

We have attempted in this work to determine if the GSH–ascorbic acid enzyme system functions as an electron-transferring system *in vivo*, and if possible to estimate the proportion of the total respiration which passes over it. As in most work of this type, it is relatively easy to construct models of such respiratory systems *in vitro* but more difficult to assess their importance *in vivo*.

In order to obtain a biochemical analysis which provides a reliable assessment of the respiration *in vivo*, the first objective must be to prepare extracts from the tissue, the respiration of which is a real index of the respiration of the intact tissue. In this work it appears that this was possible in the special case of the cotyledon of the pea seedling. It is, of course, conceivable that even here an artificial respiration is being measured, the corremean substrate concentration in the suspension may be very different from the concentrations of the same substrates in the protoplasm, due to the vacuolar contribution. For these reasons it was impossible to draw any confident conclusions about the part played by the GSH–ascorbic acid system in the respiration of the detached embryo, beyond the suggestion that the system may be operating as an electron carrier *in vivo*.

On the assumption that the oxygen consumption of the extracts from the cotyledon is a reliable index of the respiration *in vivo*, the evidence indicates that a little less than half the respiration of the soluble fraction of the cytoplasm passes over the GSH–ascorbic acid system, which is equivalent to approximately 25% of the total respiration of the cotyledon.

In a homogeneous solution, one would expect competition between different hydrogen-transfer

systems for the hydrogen passing over a common coenzyme. In such circumstances the relative affinities of different enzymes, such as TPN–cytochrome *c* reductase, glutathione reductase and diaphorase enzymes would presumably determine the extent of hydrogen transfer over either system. How far this state of affairs exists when two enzymes are localized each in a different part of the cell is difficult to say. The results provided no evidence that the rate of oxygen consumption passing over the GSH–ascorbic acid system was altered by the presence of the cytochrome system localized within the mitochondria. If the respiration of the former system had been affected, one would expect that the oxygen consumption of the soluble part of the cytoplasm, determined separately, would have been greater than when it was present with the mitochondria in the suspension. In fact, the total oxygen consumption of the mitochondria (in the presence of the substrates of the soluble fraction), and of the soluble fraction, when added together, was nearly identical with that obtained before their separation. On the other hand, there was evidence of competition between enzyme systems localized in the soluble part of the cytoplasm in extracts prepared from the detached embryo. Suppression of diaphorase enzymes oxidizing TPNH, by the exclusion of oxygen, appeared to increase the electron transfer over the GSH–ascorbic acid system, as shown by the increased rate of reduction of DHA in nitrogen relative to the rate with cyanide in air.

Apart from ascorbic acid oxidase, which only develops its full activity after the first 3 days of germination, the enzymes associated with the system are present in the dry seed and operate from the early stages of germination. In this early phase the production of GSH, and all that it implies in the activation of SH enzymes, may be of more importance than the functioning of the system in respiration; certainly its full potentiality cannot be realized until development of oxidase activity occurs.

SUMMARY

1. It has been shown with extracts prepared from germinating pea seeds that reduced glutathione and ascorbic acid act as respiratory carriers in an enzyme system which transfers hydrogen to molecular oxygen from substrates of TPN-linked dehydrogenases. The enzymes concerned in this respiratory pathway include dehydrogenase enzymes, glutathione reductase, dehydroascorbic acid reductase and ascorbic acid oxidase.

2. The enzyme dehydroascorbic acid reductase was confined to the soluble part of the cytoplasm and was absent from the mitochondria.

3. The oxygen consumption of suspensions prepared from the cotyledons was of the same order of magnitude as that of the intact tissue, but the oxygen consumption of suspensions from the detached embryo was only about one-tenth that of the intact tissue.

4. The oxygen consumption of suspensions from the cotyledons was almost equally distributed between the mitochondria and soluble fraction of the cytoplasm, but with suspensions from detached embryos the proportion of the respiration attributable to the soluble fraction decreased from 70% at 5 days to 50% after 9 days.

5. Both cyanide and diethyldithiocarbamate, which in millimolar concentrations completely inhibited the oxidation of ascorbic acid, equally depressed the respiration of the soluble fraction of the cytoplasm prepared from suspensions of the whole seedling.

6. Approximately 70% of the total oxygen consumption of the soluble fraction from the cotyledons was cyanide-sensitive and, from the rate of reduction of dehydroascorbic acid by this soluble fraction, it would appear possible that 60% of the cyanide-sensitive respiration might pass over the reduced glutathione–ascorbic acid system. This in turn suggests that 25% of the total respiration of the cotyledons may pass over this route.

7. The reduced glutathione–ascorbic acid system may also function as a respiratory system in the detached embryo but, owing to the lack of correspondence between the respiration of suspensions and intact tissue, no quantitative estimate of its importance can be given.

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Studies in Carotenogenesis

16. THE ACTION OF SOME ANTIBIOTICS, ESPECIALLY STREPTOMYCIN, ON CAROTENOGENESIS IN *PHYCOMYCES BLAKESLEEANUS**

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Bracco & von Euler (1947, 1948) observed that when barley seedlings were germinated on a medium containing streptomycin they produced white coleoptiles and first leaves which contained neither chlorophyll nor carotenoids but normal amounts of anthocyanins. Green seedlings transferred to a streptomycin medium did not lose their plastid pigments, although etiolated seedlings failed to become green or to develop chloroplasts on exposure to light in the presence of streptomycin. These findings have been largely confirmed by using *Pinus jeffreyi* (Bogorad, 1950) and *Pisum* spp. (Schopfer, Bein & Besson, 1952). The green crown gall tumours of carrots also turn white in the presence of streptomycin (Ropp, 1948).

Similar colour changes were first observed in micro-organisms by Provasoli, Hutner & Schatz (1948), using *Euglena gracilis* v. *bacillaris*; in this case there was a permanent loss of chloroplasts, the algae remaining colourless on serial transfer to streptomycin-free media in the light. This has been confirmed by Lwoff & Schaeffer (1949*a*, *b*) and Jirovec (1949); Goodwin & Jamikorn (1954) showed that although considerably reduced by streptomycin, carotenoid synthesis is less affected than chlorophyll synthesis. The whole streptomycin molecule is necessary for inhibition of pigment formation in *E. gracilis* (Provasoli, Hutner & Pinter, 1951) although dihydrostreptomycins A and B are also active (Hutner & Provasoli, 1951). Provasoli *et al.* (1951) also reported no obvious colour change in cultures of five strains of photosynthetic Athiorhodaceae, in which growth had been partly inhibited by streptomycin. Goodwin & Osman (1953) found, however, in quantitative experiments with *Rhodospirillum rubrum*, that there was a narrow range of concentration over which streptomycin inhibited carotenoid (spirilloxanthin) synthesis to a greater extent than growth; the effect was very much less marked than with *E. gracilis*.

Because of these reports the effect of streptomycin on carotenogenesis in a non-photosynthetic organism, *Phycomyces blakesleeanus*, has been examined in detail. A preliminary report of the inhibition of carotenogenesis in this organism by streptomycin has already been made (Goodwin & Griffiths, 1952) and this has been confirmed by Schopfer, Grob, Besson & Keller (1952).

Although no other antibiotic was found which had the same effect as streptomycin on *E. gracilis* (Hutner & Provasoli, 1951), it has been briefly reported that chloromycetin inhibits carotenogenesis in both pea seedlings and *P. blakesleeanus*, that tetronic acid is active only on pea seedlings and that penicillin is inactive on both organisms (Schopfer, Grob, Besson & Keller, 1952; Schopfer, Grob & Besson, 1952*a*, *b*). In the present investigation these and other antibiotics have also been examined, but in less detail than streptomycin.

EXPERIMENTAL

Cultures. Phycomyces blakesleeanus (−strain) obtained from the Centraalbureau voor Schimmelcultures, Baarn, Holland, was used throughout the investigation.

The standard medium was that described by Garton, Goodwin & Lijinsky (1951) modified to contain 2·5% (w/v) glucose. The basal acetate medium used was medium *C* of Friend, Goodwin & Griffiths (1955), based on that of Schopfer & Grob (1950, 1952).

* Part 15: Friend, Goodwin & Griffiths (1955).

The antibiotics were added to the autoclaved media (15 lb./sq.in./15 min.) either from sterile ampoules or after sterilization by filtration.

Inoculation was carried out with spore suspensions prepared and dispensed as described by Garton *et al.* (1951).

Mycelia were normally grown either in 8 oz. medicine bottles containing 15 ml. of medium or in penicillin flasks containing 100 ml. of medium, and incubated at 25° in a glass incubator (Garton *et al.* 1951). In order to obtain mats for transfer to fresh medium, mycelia were grown in Petri dishes and the method of transference used was that described by Goodwin & Willmer (1952).

Dry-weight lipid and carotene determinations. In all cases, except where otherwise stated, three cultures were combined for each analysis; this saves a considerable amount of time and is justifiable, because Lijinsky (1951) has shown that when groups of 10 cultures are examined separately the mean values of highest and lowest groups of three never differed from the mean of the 10 analyses by more than 6% in dry weight and 10% in carotene.

Dry-weight determinations of mycelia grown on glucose media were carried out by the method of Garton *et al.* (1951) and of mycelia grown on acetate by the method of Friend *et al.* (1955), which involves a slight modification of the original method owing to the precipitation in the medium of inorganic phosphates during growth. These are removed by washing the mycelium with 5 ml. of 2N-HCl. Lipid and carotene determinations were carried out in all cases using the method of Garton *et al.* (1951).

Chromatographic separation of the carotenoids was carried out with alumina as adsorbent (Goodwin, 1952).

Formation of 2:4-*dinitrophenylhydrazones.* Two methods were used, one for routine identification and one for isolation. In the first procedure, Brady's reagent was added to the medium which was then kept at 37° overnight; on cooling to room temperature, the orange-yellow precipitate was collected and dissolved in ethyl ether freshly distilled over reduced iron [if undistilled ether is used the hydrazones eventually give anomalous colours in alkaline solution (Whittam & Davies, 1954)]. The ethereal solution was then shaken in a separating funnel with an equal vol. of 5% (w/v) Na_2CO_3, which extracts acid hydrazones (Goodwin & Williams, 1952). After separation from the ether layer (*A*) the aqueous solution is immediately acidified with 2N-HCl and the acid hydrazones are extracted back into ethyl ether; considerable destruction of the hydrazones occurs if they are allowed to stand in alkaline solution for any length of time. After the ether solution has been washed free from acid with successive small volumes of water, it is ready for paper chromatography. The original ether extract (*A*) is similarly washed free from alkali preparatory to chromatography.

In the second method the pH of the medium was adjusted to 10 with 2N-NaOH and extracted with freshly distilled ethyl ether in a continuous extractor for 6 hr. The solvent was then removed from the extract *in vacuo* and the 2:4-dinitrophenylhydrazones (DNPH's) prepared from the residue in the usual manner. The medium was then taken to pH 1 with conc. HCl and again extracted with ethyl ether; the ether extract was taken to dryness *in vacuo* and the DNPH's prepared. Finally the residual medium was treated with Brady's reagent, and any precipitate formed was treated as described in the first method.

Chromatography of the 2:4-*dinitrophenylhydrazones.* The neutral DNPH's were chromatographed on paper by the ascending method, using the two-phase solvent system of Meigh (1952) in which heptane was the mobile and methanol the stationary phase. The DNPH's were applied in ether. The acid hydrazones were separated using the method of Altmann, Crook & Datta (1951), which employs a *tert.*-amyl alcohol–ethanol–water solvent system. The hydrazones were applied in a glycine/NaOH buffer (0·1M) of pH 8·2–8·4.

The yellow-orange spots could easily be located visually on the paper, and further characterized by spraying with 2N-NaOH, which produced characteristic colours varying from brown to blue. In order to measure the absorption spectrum of the separated spots they were eluted with warm glacial acetic acid, transferred to ethyl ether and washed free from acid, and the spectrum was measured. The ethyl ether extracts were then evaporated to dryness *in vacuo*, dissolved in 3 ml. of ethanol, diluted with an equal vol. of 1·5N-NaOH and the absorption spectra measured as quickly as possible.

The method of Kulonen (1953) in which the DNPH's of keto acids are reduced with Al-amalgam to the corresponding amino acids, which are then chromatographed in the conventional manner, has also been employed.

Spectral measurements. These were carried out with the Unicam SP. 600, SP. 500 or Beckman DU instruments. Measurements were made at 5 mμ. intervals, except near maxima, where the interval was reduced to 1·0 mμ. or 0·5 mμ.

RESULTS

Effect of streptomycin on carotene synthesis

Even very high concentrations of streptomycin have no marked effect on growth of 7-day cultures of *Phycomyces blakesleeanus* (Fig. 1). With β-carotene, however, there was a gradual but marked decrease in synthesis with increasing concentration of streptomycin up to a level of 0·075–0·10% (w/v), when the carotene level was 30–40% of its normal value. Further increases in streptomycin had no added effect on carotene production.

When mycelia are examined at various stages of development, it will be seen (Table 1) that streptomycin slows down growth but, confirming the results shown in Fig. 1, does not alter the amount of growth finally achieved. Inhibition of carotenogenesis does not take place in young growing mycelia. The results in Table 1 confirm the earlier observations (Garton *et al.* 1951) that carotenogenesis in the dark is only about 50% of that in the light, although growth is normal, and show that the 'dark' synthesis of β-carotene is also inhibited by streptomycin.

When the glucose concentration of the standard medium is reduced to 1% (w/v), growth is reduced to a lesser extent than is carotenogenesis (Goodwin & Willmer, 1952). The results given in Table 2 show that this reduced level of synthesis is further lowered by streptomycin. On the other hand, carotenogenesis by vegetative mats placed on a glucose/salts medium (i.e. in the absence of N) is almost completely inhibited by the antibiotic (Table 3).

It is justifiable to assume in each experiment that all mats at transfer have the same weight and contain the same amount of β-carotene (Lijinsky, 1951). Although variations between flasks in a single experiment are small, mats from experiments carried out at different times can vary considerably, especially during the early stages of growth (2–4 days). This accounts for the different basal values in the experiments quoted in Table 3.

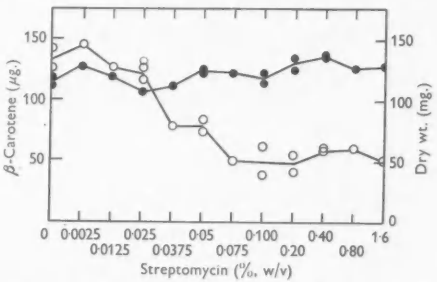

Fig. 1. Effect of streptomycin on growth and carotenogenesis by *P. blakesleeanus*. Standard glucose medium with varying amounts of streptomycin; amounts produced in 15 ml. of medium in 8 oz. medicine bottles in the light; 7-day cultures, temp. 25°. Each point represents the mean value for three bottles. •, Total dry wt.; o, β-carotene.

When asparagine in the standard medium is replaced by leucine or valine, a considerable stimulation of β-carotene synthesis results (Goodwin & Lijinsky, 1952). Table 4 shows that this stimulation is also streptomycin-sensitive. When asparagine is replaced by NH_4NO_3 or $(NH_4)_2SO_4$ there is a considerable reduction of growth and of carotenogenesis (Garton *et al.* 1951). This change in the nitrogen source, however, results in almost complete elimination of the streptomycin effect on carotene synthesis (Table 5). Even the presence of NH_4NO_3, together with asparagine, reduces the sensitivity of the carotenogenic system to streptomycin. The final pH of the media are recorded for these experiments because it was possible that the slightly lower values on the NH_4NO_3 media might have affected the action of the antibiotic (see Discussion).

In 1940, Leonian & Lilly showed that the addition of a number of carboxylic acids (0·02M) to the glucose/NH_4NO_3 medium considerably

Table 1. *Effect of streptomycin on growth and β-carotene synthesis by* Phycomyces blakesleeanus *at various times after inoculation*

Standard medium; amounts produced in 15 ml. of medium in 8 oz. medicine bottles; temp. 25°; two bottles bulked for each analysis.

Age of culture ...	3 days			5 days			7 days		
		β-Carotene			β-Carotene			β-Carotene	
	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
Light cultures									
1. Standard medium	46·3	12·3	266	105·2	88	837	106·3	115	1083
2. 1+0·4% (w/v) streptomycin	21·4	7·5	351	121·8	56	460	110·4	60	544
Dark cultures									
1. Standard medium	52·1	6·3	120	115·1	45	400	120·0	61	510
2. 1+0·4% (w/v) streptomycin	30·2	7·6	250	128·1	31	240	129·0	41	360

Table 2. *Effect of streptomycin on β-carotene synthesis by* Phycomyces blakesleeanus

Standard medium with glucose concn. reduced from 2·5 to 1·0% (w/v); amounts produced in 15 ml. of medium in 8 oz. medicine bottles; temp. 25°; 7-day cultures in the light; three mycelia bulked for each analysis.

Expt. no.	Streptomycin absent			Streptomycin present (0·2%, w/v)			Inhibition of β-carotene production by streptomycin (%)
		β-Carotene			β-Carotene		
	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	
1	76·5	19·4	253	95·6	11·0	115	43·3
2	57·2	10·7	187	59·2	7·7	130	28·0
3	49·4	21·5	435	62·3	10·1	160	53·0
4	32·9	14·3	435	41·6	6·7	161	53·2

stimulated growth of *P. blakesleeanus*. This has been confirmed by Friend *et al.* (1954, 1955), who also showed that the accompanying stimulation of carotenogenesis varied with the acid used; this is due to variations in the final pH of the different media, the greatest stimulation at a level of 0·02M being obtained with acetate and succinate. A large number of experiments have been carried out on the effect of streptomycin on growth and carotenogenesis by *P. blakesleeanus* on the glucose/NH_4NO_3 medium in the presence of different carboxylic acids. In no case was there an effect on

Table 3. *Effect of streptomycin on β-carotene synthesis by vegetative mats of* Phycomyces blakesleeanus

3-day mats grown in Petri dishes containing 15 ml. of standard medium with glucose concn. reduced to 1% (w/v), washed with sterile water and transferred to 15 ml. standard medium without asparagine in the presence or absence of 0·2% (w/v) streptomycin. 2 days incubation at 25° in the light; three mats bulked for controls and 'normal' transfers, six mats for streptomycin transfers.

	β-Carotene (μg.)			
	Amount at transfer	Amount after transfer	Increment	Inhibition (%)
Expt. A				
Without streptomycin	135*	264	129	—
With streptomycin		156	21	83·7
Expt. B				
Without streptomycin	41*	88	47	—
With streptomycin		42·4	1·4	97·1

* The different amounts of carotene present in the two cases is due to the different weights of the mats when harvested, the mean values being 108 and 47 mg. respectively.

Table 4. *Effect of streptomycin on leucine-stimulated carotenogenesis in* Phycomyces blakesleeanus

Amounts produced on 15 ml. of standard medium containing 1% (w/v) glucose and with either L-asparagine or DL-leucine (0·2%, w/v) as N source; concn. of streptomycin 0·2%, (w/v); temp. 25°; three mycelia bulked for each determination.

	7-day cultures			11-day cultures		
		β-Carotene			β-Carotene	
Nitrogen source	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
Without streptomycin						
Asparagine	32·9	14·3	435	43·2	9·5	220
Leucine	19·9	16·3	820	37·2	49·9	1341
With streptomycin						
Asparagine	41·6	6·7	161	58·8	3·0	51
Leucine	21·5	10·0	465	38·3	33·5	875

Table 5. *Effect of streptomycin on carotenogenesis in* Phycomyces blakesleeanus *cultured on various nitrogen sources*

Amounts produced in 8 oz. medicine bottles containing 15 ml. of standard medium with asparagine replaced by different N sources; streptomycin 0·2% (w/v); 7-day cultures; temp. 25°. Three mats combined for each analysis.

		Without streptomycin				With streptomycin			
			β-Carotene				β-Carotene		
Nitrogen source	Concn. (%, w/v)	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	pH of medium	Dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Final pH of medium*
Asparagine	0·20	94·5	128	1356	4·5	118·2	33·3	282	4·4
NH_4NO_3	0·25	17·7	26·2	1482	3·3	24·7	36·9	1498	2·8
$(NH_4)_2SO_4$	0·25	13·7	34·2	2500	3·7	20·1	40·7	2025	3·1
NH_4NO_3 and asparagine	0·175 / 0·075	37·7	105	2785	2·6	52·1	106	2035	2·6
NH_4NO_3 and asparagine	0·25 / 0·20	79·8	143	1792	3·7	86·9	114	1312	3·4

* All media were originally adjusted to pH 6·0.

growth. The effect on carotenogenesis is illustrated in Table 6, where the mean carotene production in the presence of streptomycin in each separate experiment is represented as a percentage of that produced at the same time in the absence of streptomycin. Marked and consistent inhibition only occurs when succinate is the stimulatory carboxylic acid. Even when carotene production is increased in the presence of malate, fumarate, etc. by reducing the concentration of these acids to 0·01M (Friend *et al.* 1955) no marked streptomycin inhibition was observed.

containing medium. Fig. 2 shows that growth and carotenogenesis are both affected equally, so that (except with very high concentrations of streptomycin which reduces growth so considerably that accurate analyses are impossible) the concentration of carotene does not alter; the lipid levels followed the same pattern and are not recorded. The same results are obtained when yeast extract (0·1%), which stimulated growth and carotenogenesis on an acetate medium (Friend *et al.* 1954, 1955), is added to the acetate medium; they are, therefore, not reproduced here.

Table 6. *Effect of streptomycin on carotenogenesis by* Phycomyces blakesleeanus *on a glucose/NH_4NO_3 medium, in the presence of various carboxylic acids*

Results recorded as percentage of control value in same experiment; three bottles bulked for each determination; 7-day cultures; streptomycin 0·2% (w/v); temp. 25°.

Carboxylic acid added	Concn. (M)	Amount of β-carotene (% of control)	Final pH of medium (mean) Without streptomycin	With streptomycin
None	—	98, 113	3·3	2·8
Acetate	0·02	113, 121, 106, 105	2·4	2·4
Succinate	0·02	53, 51, 64, 51	2·7	2·8
Citrate	0·02	77, 82, 133	4·0	4·0
	0·01	96, 80	3·3	3·1
α-Oxoglutarate	0·02	88, 92	2·8	3·3
	0·01	86	3·2	4·4
Fumarate	0·02	109, 91	3·5	2·6
	0·01	115, 72	3·2	4·4
Malate	0·02	97, 90, 116	2·5	3·0
	0·01	82	2·9	2·8

Table 7. *Effect of streptomycin on β-ionone-stimulated carotenogenesis in* Phycomyces blakesleeanus

Amounts produced in 100 ml. of standard medium in penicillin flasks: β-ionone 10 μl./flask; streptomycin concn. 0·2%, (w/v); 7-day cultures; temp. 25°; two flasks bulked for each analysis.

	Normal cultures	β-Ionone cultures	β-Ionone + streptomycin cultures
Total dry wt. (mg.)	477	467	581
β-Carotene (μg.)	672	1300	360
β-Carotene concentration (p.p.m.)	1409	2780	620

Effect of streptomycin on β-ionone stimulation

Mackinney, Nakayama, Buss & Chichester (1952) found that addition of small amounts of β-ionone to glucose/asparagine cultures of *P. blakesleeanus* considerably stimulates carotenogenesis. The results given in Table 7 confirm this finding, and also demonstrate that this stimulation is streptomycin-sensitive.

Effect of streptomycin on carotenogenesis by Phycomyces blakesleeanus *on acetate media*

The effect of streptomycin on *P. blakesleeanus* grown on a medium containing acetate as the sole carbon source is different from that on a glucose-

Effects of various degradation products of streptomycin on carotenogenesis

The results of a small number of experiments carried out with the limited quantities available to us of some fragments of the streptomycin molecule (Table 8) show that methyl streptobiosaminidine dimethyl acetal inhibits carotenogenesis in *P. blakesleeanus*. All the other fragments examined were inactive.

Attempts to reverse the inhibition of carotenogenesis by streptomycin

Loo, Carter, Kehm & Anderlik (1950) reported that fructose 1:6-diphosphate reversed the streptomycin inhibition of growth in *Lactobacillus*

arabinosus, *Staphylococcus aureus* and the yeast *Torula utilis*. At the concentration recommended by Loo *et al.* (1950) (0·512%, w/v), this compound had no effect on the inhibition of carotenogenesis by streptomycin. Similarly, pantothenic acid, reported to reverse the growth inhibition of streptomycin in *Saccharomyces fragilis* (Lichstein & Gilfillian, 1951), was without effect at levels of up to 200 mg./100 ml. Further, neither of these two compounds had any effect on growth or carotenogenesis by *P. blakesleeanus* in the absence of streptomycin, although, in agreement with the observations of Hawker (1948), it was found that production of sporangiophores occurred sooner in the presence than in the absence of hexosediphosphate.

Streptomycin and the production of carbonyl compounds by Phycomyces blakesleeanus

The possibility that the building unit for carotenogenesis may be an aldehyde such as β-methylcrotonaldehyde or *iso*valeraldehyde (Goodwin, 1953), and that these might accumulate in the presence of streptomycin, prompted an investigation into the carbonyl compounds present in the normal and streptomycin media.

Neutral carbonyl compounds. The 2:4-dinitrophenylhydrazones (DNPH's) which are not extracted from ethyl ether by Na_2CO_3 were chromatographed with the solvent system of Meigh (1952); only one spot was obtained which remained at the origin. It gave a blue coloration with 3N-NaOH indicating a dicarbonyl compound; glucose,

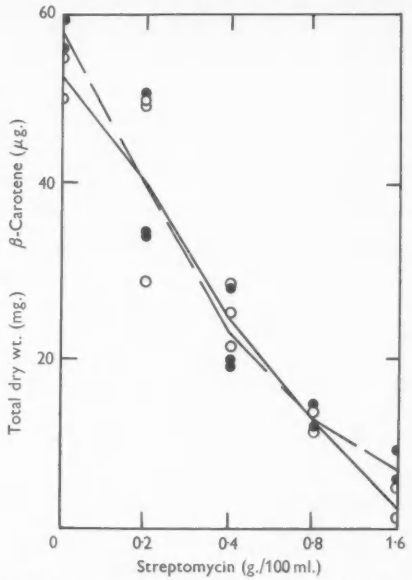

Fig. 2. Effect of streptomycin on growth and carotenogenesis by *P. blakesleeanus*. The all-acetate medium with varying amounts of streptomycin. Amounts produced in 100 ml. of medium in penicillin pots in the light; temp. 25°; 7-day cultures. Each point represents the mean value of two pots bulked for analysis. •, Total dry wt.; o, β-carotene.

Table 8. *Effect of various fragments of the streptomycin molecule on carotenogenesis by* Phycomyces blakesleeanus

Amounts produced in 8 oz. medicine bottles containing 15 ml. of medium; 7-day cultures; temp. 25°. Concn. of inhibitor 0·1% (w/v); three experiments carried out at different times; three bottles bulked for each analysis.

Fragment	Expt.	Dry wt. (mg.)	β-Carotene Amount (μg.)	β-Carotene Concn. (p.p.m.)
Control	A	98·8	87	881
	B	100·5	91	903
	C	103·2	90	872
Streptobiosaminidine dimethyl acetal	A	91·3	39	428
	B	92·4	41	454
	C	90·0	36	402
L-*N*-Methylglucosamine	A	99·9	77·5	837
	B	95·5	80	768
	C	103·2	79	883
Streptosamine	A	91·2	80·5	883
Streptidine sulphate	A	88·3	87	981
	B	86·6	88	1016
	C	87·0	86	988
Streptamine hydrochloride	B	92·5	72	778
	C*	89·0	90	998

* Only two determinations.

dihydroxyacetone and glyceraldehyde were ruled out because they form DNPH's only on boiling, also all their DNPH's except that of dihydroxyacetone, exhibited different absorption spectra from that of the unknown. The remaining possibilities were methylglyoxal and glyoxal; the latter was ruled out because of the different absorption spectrum of its DNPH (Fig. 3), while the spectrum

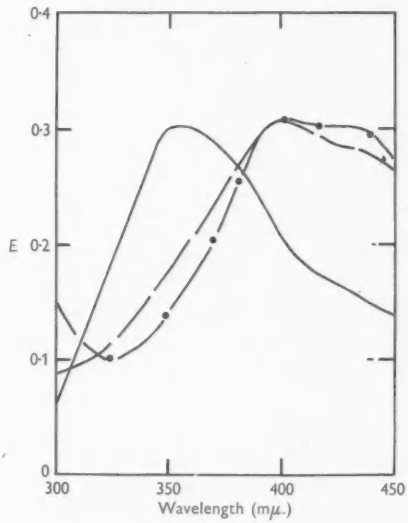

Fig. 3. Absorption spectra in ether of the 2:4-dinitrophenylhydrazones of: methylglyoxal, – – –; glyoxal, ——; neutral metabolite from culture medium of *P. blakesleeanus*, —•—•—. The $E_{max.}$ values have been adjusted to the same level for the three compounds.

of the unknown DNPH was almost identical with that of methylglyoxal. All the compounds gave the same absorption spectrum with a broad maximum at 560 mμ. when treated with NaOH. As the unknown was present in the medium only in minute traces, it was not possible to obtain it crystalline, but the above evidence makes it almost certain that it is methylglyoxal.

The acid carbonyl fraction. Chromatography of the acid DNPH's isolated by methods A (Experimental) and by using the solvent system of Altmann *et al.* (1951) revealed three spots *A*, *B* and *C*, with R_F values of 0·36, 0·75 and 0·80 respectively. *A* and *C* occurred only in traces. Compound *A* was shown to be the DNPH of pyruvic acid by the identity of its absorption spectrum in ethanol and 1·5N-NaOH with that of an authentic specimen (Goodwin & Williams, 1952) and by its failure to separate from an authentic specimen of pyruvic acid DNPH on paper chromatography of the mixture. Further, in media deficient in thiamine, the intensity of this spot increased very considerably, and Haag (1940) has found that the extracellular pyruvate produced by a number of yeasts and moulds was very much increased when these organisms were grown in the presence of suboptimal amounts of thiamine.

Spot *C* was yellowish brown and gave a blue coloration with NaOH; because of the small amounts produced, it was not investigated further. The major spot, *B*, was khaki-coloured and gave a bright purple-blue colour with alkali. When the keto acids were extracted as such from the medium (with ethyl ether at pH 1), the DNPH's produced did not contain fraction *B*. Subsequent treatment of the residual medium with DNPH produced compound B. In streptomycin media, *B* could easily be separated from the DNPH of streptomycin by extracting the treated medium with ethyl ether, in which the latter is insoluble. From 2 l. of medium (20 penicillin flasks) about 6 mg. of crystalline *B* was obtained which decomposed above 350° without melting and contained 20·3% of N (≡mol.wt. of 178 assuming a bis-derivative). The absorption maxima of its spectrum in ethyl ether and 0·75N-NaOH were at higher wavelengths than any of those recorded for the DNPH's of the keto acids derived from the naturally occurring amino acids (Cavallini, 1950). Treatment of the DNPH with aluminium amalgam, with subsequent paper separation of the products (Kulonen, 1953), produced three spots which fluoresced in ultraviolet light with colours varying from bright orange to bright green; none of the spots, however, was ninhydrin-positive, thus confirming the supposition that *B* was not a DNPH of a keto acid.

The possibility that *B* was the DNPH of an enediol was examined by comparing its absorption spectrum in ether and NaOH with those of a number of such or related compounds. Its spectral properties correspond very closely in position (in both ethyl ether and 0·75N-NaOH) and shape with that of hydroxytetronic acid but are quite different from those of the other related compounds examined (Table 9). A mixed paper chromatogram of the DNPH's of the metabolite and hydroxytetronic acid showed that the two compounds are easily separable, indicating their non-identity. It would seem, however, that the metabolite is probably a derivative of tetronic acid but that it is not ascorbic acid.

Neither of these two metabolites could be detected when vegetative mats were dissimilating glucose or when the fungus was grown on an acetate medium.

Table 9. *Absorption spectrum maxima (mμ.) of 2:4-dinitrophenylhydrazones of a number of ene-diols*

	Solvent	
	Ethyl ether (mμ.)	0·75 N-NaOH (mμ.)
Metabolic product of *P. blakesleeanus*	395, 430	535
Hydroxytetronic acid	390, 435	535
Terrestric acid	340	449
Penicillic acid	357	410
Carlosic acid	315	412
Carlic acid	343	417
Carolic acid	341	413
Carolinic acid	343	415
*iso*Ascorbic acid	348	420
Ascorbic acid	343	520, 544
Reductone	348, 345	Destroyed in alkali
Reductic acid	370	Destroyed in alkali

Table 10. *Effect of various antibiotics on growth and carotenogenesis in* Phycomyces blakesleeanus

Amounts produced in 8 oz. medicine bottles containing 15 ml. of standard medium; three mycelia bulked for each analysis; 7-day cultures; temp. 25°.

Antibiotic added	Concn. (%, w/v)	% of control values	
		Dry wt.	β-Carotene
Chloromycetin	0·02	103·1, 968	90·9, 95·1
	0·10	77·7, 843	44·6, 46·7
	0·16	84·2	27·9
	0·20	46·3	12·4
Aureomycin	0·001	106·0	105·9
	0·01	Complete inhibition	
Penicillin	0·02	95·7	129·2
	0·16	Complete inhibition	
Heximide	0·000015	114·2	49·7
	0·00015	82·0	69·1
	0·0010	88·9	49·7
	0·0015	80·1	52·6
	0·020	79·8	68·7
	0·050	Complete inhibition	
Terrestric acid	0·010	90·7, 100·5	53·9, 85·7
	0·015	35·4	28·6
	0·020	57·4	32·7
	0·025	Complete inhibition	
Penicillic acid	0·010	95·9	86·6
	0·025	100·6, 86·8	58·2, 26·6
	0·05	47·0	9·9
	0·10	Complete inhibition	
Carlosic acid	0·025	112·6	86·7
	0·25	Complete inhibition	
Carlic acid	0·025	111·9	134·0
	0·25	Complete inhibition	
Carolic acid	0·025	78·9	86·0
	0·25	Complete inhibition	
Carolinic acid	0·01	99·1	99·4
	0·025	87·3	72·0
	0·05	83·5	62·1
	0·10	Complete inhibition	

Effect of streptomycin on the production of methylglyoxal and the ene-diol

It was always obvious from visual inspection of chromatograms of the DNPH's of 'streptomycin media' that *B* was almost completely absent and that the amount of methylglyoxal-like compound was reduced. In a number of experiments the ene-diol was determined quantitatively as for pyruvate (Goodwin & Williams, 1952), using 560 mμ. instead of 412 mμ. for determining the *E* value; the methylglyoxal was similarly determined in the ethyl ether extract after removing the ene-diol with 5% (w/v) Na_2CO_3. Comparison of the normal and streptomycin media showed that in the latter the synthesis of *B* was reduced on the average by 93% and that of methyglyoxal by 58%.

Effect of antibiotics other than streptomycin

A number of other antibiotics have been reviewed for their effect on growth and carotenogenesis in *P. blakesleeanus*. Table 10 shows that chloramphenicol, penicillic acid and terrestric acid inhibit carotenogenesis to a much greater extent than they do growth, while aureomycin, penicillin, and carlosic, carlic, carolic and carolinic acids did not have a marked effect on either growth or carotenogenesis at concentrations below those at which they inhibit growth completely. It has not been possible to examine some of the acids in any further detail because of lack of material.

The results with chloramphenicol and with penicillin confirm those of Schopfer and his group.

DISCUSSION

Mode of action of streptomycin

Carotenogenesis in *P. blakesleeanus* cultured on the standard medium can be divided into two phases: (*a*) about 30% of the total, produced during the growing period, and (*b*) about 70% of the total, produced after growth is completed (Goodwin & Willmer, 1952). The results obtained with streptomycin would justify the working hypothesis that the antibiotic inhibits the second phase only, for the following reasons: (i) the maximal inhibition of carotenogenesis on the standard medium is about 60% (Fig. 1), (ii) carotene synthesis by vegetative cells (phase b) is almost completely inhibited, (iii) no inhibition occurs in young cultures (Table 1) and (iv) in the absence of excess of glucose (i.e. when the concentration is reduced from 2·5 to 1·0%) the degree of inhibition is perhaps slightly reduced. (In this case the reduction is not as clear-cut as might be expected, but it is likely that phase b is proceeding to some degree even during growth.) The main difference between the two phases might be that during growth, asparagine is also utilized

for carotene synthesis whilst after growth is completed glucose only is used, a conclusion compatible with the observations of Chichester, Mackinney & Wong (1954) on the incorporation of labelled fructose into β-carotene by *P. blakesleeanus*. When the effects of streptomycin on the acetate and the glucose/asparagine media are compared, at least three possible interpretations exist: (A) That as growth and carotenogenesis on the acetate medium are equally inhibited, the primary effect is on growth. This would lead to the conclusion that neither growth nor the streptomycin-sensitive synthesis of β-carotene on the glucose/asparagine medium proceeds via acetate. For general anabolic processes (growth) this conclusion would appear unlikely and thus it may be that streptomycin acts primarily by inhibiting the activation of acetate. (B) If streptomycin were not directly affecting carotenogenesis on the acetate medium, then the acetate not being utilized for growth could theoretically be 'shunted' into carotene synthesis so that the concentration would increase. As this is not the case, it could be concluded that acetate incorporation into β-carotene is directly affected by streptomycin and that the streptomycin-sensitive carotene synthesis on the glucose/asparagine medium proceeds via acetate. (C) As the final pH of the acetate medium is about 7·8, which is near the optimum for streptomycin activity against bacteria (Henry, Henry, Berkman & Housewright, 1947; quoted by Florey *et al.* 1949), an effect is apparent which cannot be produced at a lower pH (glucose medium); this implies that carotenogenesis is more sensitive than is growth to streptomycin at the lower pH. A decision as to which is correct cannot be made at present and, indeed, the situations envisaged may be oversimplified. For example, when asparagine in the standard medium is replaced by an ammonium salt, streptomycin inhibition of carotenogenesis disappears. This could be ascribed to the lower final pH obtained on the ammonium salt medium (Table 5), for there are many reports of the reduced antibacterial activity of streptomycin at low pH values; for example, it is about sixty times more active against *Staph. aureus* at pH 7·7 than at pH 5·2 (Wolinsky & Steenken, 1946). It is unlikely that streptomycin is destroyed at the lower pH prevailing in the ammonium salt media, for Regna, Wasselle & Solomons (1946) showed that no appreciable hydrolysis of streptomycin occurred over 900 hr. at pH 1·7.

The failure of streptomycin also to inhibit the increased carotene production on the glucose/NH_4NO_3 medium in the presence of traces of carboxylic acids would probably be similarly explicable except for one outstanding fact: stimulation of carotenogenesis in the presence of succinate is inhibited by streptomycin. Until more is known of the mechanism of this stimulation on the glucose/NH_4NO_3 medium by the carboxylic acids, it is possible to do no more than emphasize this important anomaly.

It is important in conclusion to point out that the concentration of streptomycin required to produce maximal inhibition of carotenogenesis in *P. blakesleeanus* is much higher than that used in studying bacterial inhibition (0·1–0·2%, (w/v), compared with 0·5–50 μg./ml.) and that required to produce permanent apochlorosis in *E. gracilis* (40–500 μg./ml. according to strain) (Hutner & Provasoli, 1951). Inhibition of carotenogenesis in the photosynthetic bacterium *Rsp. rubrum* is also achieved at a much lower concentration (10–100 μg./ml.) (Goodwin & Osman, 1953), although the effect is very much less marked than in *P. blakesleeanus*.

Methylglyoxal and the ene-diol

Although the synthesis of these two compounds is reduced in the presence of streptomycin, it is unlikely that they are connected with carotenogenesis, for neither is produced in detectable amounts when β-carotene is being synthesized either on acetate media or by vegetative cells of *P. blakesleeanus*.

Other antibiotics

Perhaps the most interesting observation emerging from the brief survey of the action of other antibiotics on carotenogenesis in *P. blakesleeanus* is that heximide inhibits carotenogenesis, although it has been reported to increase the number of yellow (carotene-containing) colonies in cultures of *Allomyces arbuscula* (Whiffen, 1952) and *Blastocladiella* spp. (Cantino & Hyatt, 1953).

SUMMARY

1. On a glucose/asparagine medium streptomycin inhibits carotenogenesis but not growth. Maximal inhibition is obtained with 0·05–0·1% (w/v) streptomycin; increased concentrations of streptomycin up to 1·6% (w/v) do not further affect carotenogenesis. Carotenogenesis in the dark and the carotenogenic action of leucine and β-ionone are also streptomycin-sensitive.

2. Replacement of asparagine in the glucose/asparagine medium wholly or partly by an ammonium salt results in failure of streptomycin to inhibit carotenogenesis. Stimulation of growth and carotenogenesis on the glucose/NH_4NO_3 medium by (0·02M) carboxylic acids is unaffected by streptomycin, with the exception of carotene stimulation in the presence of succinate.

3. Streptobiosamidine dimethyl acetal, but no other component of the streptomycin molecule, inhibits carotenogenesis.

4. On a medium in which acetate replaces glucose, streptomycin inhibits growth and carotenogenesis equally.

5. Two carbonyl compounds occur in the *P. blakesleeanus* medium; one is almost certainly methylglyoxal and the other is an ene-diol related to hydroxytetronic acid. The synthesis of the ene-diol is almost completely suppressed in the presence of streptomycin and that of methylglyoxal reduced.

6. A brief survey of the effect on carotenogenesis of a number of other antibiotics is recorded.

We are grateful to Dr D. J. Hockenhull (Glaxo Laboratories Ltd.) for generous gifts of streptomycin and its components and also of heximide, to Professor H. Raistrick, F.R.S., for the tetronic acids and related compounds, to Dr J. H. Birkinshaw for an authentic specimen of the bis-DNPH of methylglyoxal, to Dr F. Wokes (Ovaltine Research Laboratories) for samples of reductone, reductic acid and *iso*ascorbic acid, to the Medical Research Council for a grant towards laboratory expenses (T. W. G.), and to the University of Liverpool for a Fellowship (L. A. C.).

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Studies in Carotenogenesis

17. THE CAROTENOIDS PRODUCED BY DIFFERENT STRAINS OF *MYCOBACTERIUM PHLEI*

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Chargaff (1930) first demonstrated the presence of carotenoids in *Mycobacterium phlei*, and since then various strains of this organism have been investigated by Chargaff & Dieryck (1932), Ingraham & Steenbock (1935); Takeda & Ohta (1939, 1940); Turian (1950*a*, *b*, *c*, 1951, 1953) and Turian & Haxo (1952). The carotenoids reported to be present include α-, β- and γ-carotenes, a 'lycopene-like' pigment, cryptoxanthin, a 'cryptoxanthin-like' pigment, zeaxanthin, and an acidic carotenoid, chrysophlein. The acidic carotenoid astaxanthin is said to be produced by various species of Mycobacteriaceae, excluding *Myco. phlei*, when grown on hydrocarbon oils but not when grown on agar (Haas & Bushnell, 1944). Leprotene, a pigment with an absorption spectrum similar to that of β-carotene but with a greater adsorptive power and without vitamin A activity, has also been isolated. The name derives from the fact that it was first isolated from an organism considered to be *Myco. leprae* (Grundmann & Takeda, 1937), but later shown to be a strain of *Myco. phlei* (Takeda & Ohta, 1939). It has also been found in *Myco. tuberculosis* (Takeda & Ohta, 1944) and *Myco. bruyhoghe* et *adant* (Lederer, personal communication) and would appear to be a characteristic pigment of the Mycobacteriaceae. Recently, however, it has been found in an unidentified organism which does not appear to be a typical *Myco. phlei* (Goodwin & Jamikorn, 1956).

Ingraham & Steenbock (1935) first studied the factors controlling pigment synthesis in *Myco. phlei*, and found that replacement of glucose by glycerol or addition of either alcohols (particularly *iso*propanol) or glycols to the glucose media increased pigmentation. This was due mainly to the synthesis of an unidentified pigment which was similar to phthiocol isolated from *Myco. tuberculosis* by Anderson & Newman (1933); however, there was at the same time increased production of carotenoids. In contrast to the carotenoids, which reached their maximal levels before the cells began to autolyse, this pigment appeared to be an end-product of metabolism for it continued to be formed long after autolysis had set in. Later Darzins (1939) observed qualitatively that addition of olive oil increased, whilst exposure to the vapours of pine oil decreased pigmentation in *Myco. phlei*.

More recently, Turian (1950*b*) found that carotenogenesis could be separated from growth in *Myco. phlei*; ferric ions stimulated both growth and carotenogenesis equally and manganous ions specifically stimulated carotenogenesis. Developing the original observations of Kharasch, Conway & Bloom (1936) that chromogenesis in many bacteria and fungi was inhibited by diphenylamine, Turian (1950*c*) showed that this substance inhibits carotenogenesis in *Myco. phlei* without affecting growth; the inhibition was reversed by ferric ions (0·1 mg./100 ml.) but by no other cation tested. Turian (1951) further observed that phenol (1:2000–1:5000) also inhibited carotenogenesis but less effectively than did diphenylamine; resorcinol was two to three times less active than diphenylamine, whilst α-naphthylamine, thiourea, potassium cyanide and salicylaldoxime were almost inactive. Dinitrophenol, on the other hand, stimulated carotenogenesis. Turian & Haxo (1952) showed that inhibition of carotenogenesis in *Myco. phlei* by diphenylamine is accompanied by a stimulation of synthesis of the related colourless polyene, phytofluene, and a partly saturated carotene reminiscent of flavacene (Tischer, 1938, 1939).

Turian (1953) has recently reported that the incubation temperature has an important effect on carotenogenesis in *Myco. phlei*. At 30° very little chrysophlein was produced although the neutral carotenoids were formed in considerable amounts; at 37° the situation was reversed.

The present paper reports an investigation into the carotenoids elaborated by three strains of *Myco. phlei* and some factors controlling their synthesis.

EXPERIMENTAL

Cultures. The strains of *Myco. phlei* investigated were Crottin and Fleole, kindly supplied by Dr A. Lutz (Strasbourg), and Haudroy (Lausanne), kindly supplied by Dr G. Turian (Lausanne). In this paper, the latter will be called Turian's strain for convenience.

Culture conditions and media. The standard solid medium used, on which cultures were also maintained, contained (all w/v) peptone, 1%; glucose, 2%; glycerol, 2%; agar, 1·8%. The four strains were subcultured monthly.

The standard liquid medium used contained (per l.): asparagine, 5·0 g., Na_2HPO_4, 4·0 g., KH_2PO_4, 3·0 g., $MgSO_4,7H_2O$, 1 g., Na citrate,H_2O, 2·5 g., ferrous ammonium sulphate, 0·01 g., glucose, 20 g. and thiamine 0·4 mg. All the constituents of the media, except ferrous ammonium sulphate, were sterilized together by autoclaving (15 lb./sq.in./15 min.); the latter was sterilized separately by filtration and added to the autoclaved medium. The medium (100 ml.) was dispensed in 250 ml. Erlenmeyer flasks and incubated in the dark at 37°.

Analytical methods. Dry-weight determinations were carried out on the centrifuged cell mass according to the method described for the fungus *Phycomyces blakesleeanus* (Garton, Goodwin & Lijinsky, 1951). The carotenoids were also extracted according to the method of Garton *et al.* (1951) and determined as 'total carotenoids' by measuring the E value of the extract in light petroleum (light petroleum b.p. 40–60° was used throughout this investigation) at 450 mμ. and assuming the $E^{1\%}_{1\,cm.}$ (450 mμ.) for the mixed pigments to be 2500.

Separation of the pigments. The pigment extract was first saponified according to the method of Goodwin & Morton (1946) in order to remove the considerable quantities of lipid which were present and which would probably have interfered with the chromatographic separation. The resulting unsaponifiable extract was then chromatographed on active alumina (P. Spence and Co., Widnes, Grade 'O'), or alumina deactivated with methanol (Goodwin & Srisukh, 1949), or an appropriate mixture of the two, or $CaCO_3$ (analytical grade; British Drug Houses Ltd.). The chromatographic methods followed were those of Goodwin (1952*a*, 1953) and Goodwin, Jamikorn & Willmer (1953).

Production of the leprotene–iodine adduct was carried out by the method of Kuhn & Lederer (1931).

RESULTS AND DISCUSSION

Pigments present in the various strains

In order to obtain maximal amounts of pigments for chromatographic analysis, the various strains were cultured on the standard solid medium for 10 days. The same pigments are also produced in the same concentrations on the standard liquid medium; but as growth is heavier on the solid medium, the yield of pigments is consequently much greater.

As the three strains produced the same pigments a typical chromatogram is recorded for only one strain (Fleole). The first Zone (*A*) of the first separation (Table 1) on deactivated alumina consisted of a mixture of pigments and was therefore rechromatographed on a mixture (4:1) of activated and deactivated alumina (Table 2). *A* 1 and *A* 2 had properties identical with β-carotene and ζ-carotene respectively, and this was confirmed by comparison in mixed chromatograms with authentic specimens of these pigments obtained from *Phycomyces blakesleeanus* (Goodwin, 1952*a*). *A* 4 was similarly shown to be lycopene by comparison with an authentic specimen from the petals of *Calendula officinalis* (Goodwin, 1954). Although *A* 3 was not obtained in crystalline form, it is almost certainly leprotene; it has an absorption spectrum very similar to that of β-carotene, but is adsorbed more strongly on a column and, on mixed chromatography with an authentic specimen of β-carotene, the two components could easily be separated. The original investigators of leprotene (Grundmann & Takeda, 1937; Takeda & Ohta, 1939) stated that its absorption spectrum was the same as that of β-carotene. By means of the more precise spectrophotometric methods now available it has been found that the absorption spectrum of leprotene is shifted slightly to longer wavelengths compared with that of β-carotene. This is demonstrated in light petroleum in Fig. 1. The absorption

Table 1. *Chromatographic separation of the carotenoids of* Mycobacterium phlei *strain Fleole*

Adsorbent deactivated alumina; developer light petroleum containing varying amounts of diethyl ether; zones in order of increasing adsorptive power.

Zone	Description	Absorption maxima light petroleum (mμ.)	Concn. of ether (%, v/v) required for elution
A	Yellow	—	1
B	Yellow	448–452	10–20
C	Brownish yellow	428, 452	25–50
D	Brown trace	430–440*	50
E	Brown trace	428*	80–100

* Very ill-defined maxima.

Table 2. *Separation of fraction A (Table* 1*) of carotenoids of* Mycobacterium phlei *strain Fleole*

Adsorbent activated + deactivated alumina (4:1); developer light petroleum containing varying amounts of diethyl ether; pigments in order of increasing adsorptive power.

Zone	Description	Absorption maxima (mμ.) (in light petroleum)	Concn. of ether (%, v/v) required for elution	Identification
A 1	Yellow-brown	425, 450, 475	5	β-Carotene
A 2	Lemon yellow	380, 401, 422	5–10	ζ-Carotene
A 3	Brownish	428, 452, 479	10–15	Leprotene
A 4	Pink	443, 470, 500	25	Lycopene

spectrum of leprotene in various other solvents is given in Fig. 2, because the full curves have not previously been recorded in detail; the exact values of the absorption maxima are given in Table 3. The iodine adduct of leprotene was formed, using the method of Kuhn & Lederer (1931) for β-carotene, and then decomposed with $Na_2S_2O_3$. Chromatography of the regenerated pigment on a mixture of activated and deactivated alumina (4:1) revealed two zones; the major zone was unchanged leprotene (65–70% of the total), whilst the second pigment was slightly less strongly adsorbed and had its absorption maxima shifted to slightly lower wavelengths compared with leprotene (426, 450 and 475 mμ. in light petroleum). It appears to be a *cis*-isomer of leprotene. No pigment which might have corresponded with dehydro-β-carotene was detected. This reaction, therefore, represents a further means of differentiating leprotene from β-carotene, for the latter when treated with iodine is converted into a mixture of stereoisomers of dehydro-β-carotene (*iso*carotene) (Kuhn & Lederer, 1931; Zechmeister & Wallcave, 1953).

From their adsorptive properties and the positions of their main maxima fractions *B* and *C* (Table 1) are almost certainly the pigments considered by previous workers to be cryptoxanthin and zeaxanthin, respectively. The absorption spectra of the two pigments are compared in Fig. 3 with that of zeaxanthin (the spectrum of cryptoxanthin is indistinguishable from that of zeaxanthin). It is apparent that these pigments cannot be either zeaxanthin or cryptoxanthin. Recently Turian (1953) has reported a pigment 'related to cryptoxanthin' in his strain of *Myco. phlei*; it has properties identical with those of pigment *B*.

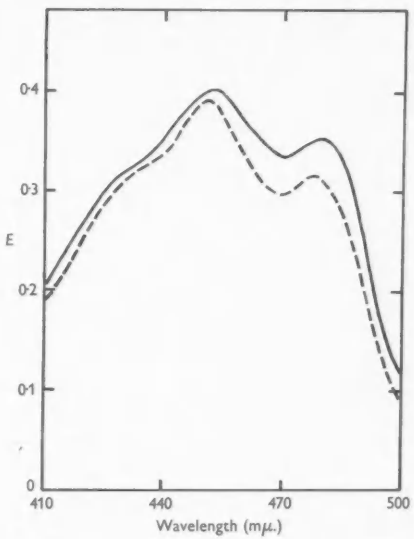

Fig. 1. Comparison of the absorption spectra of β-carotene and leprotene in light petroleum. β-Carotene, – – –; leprotene, ——.

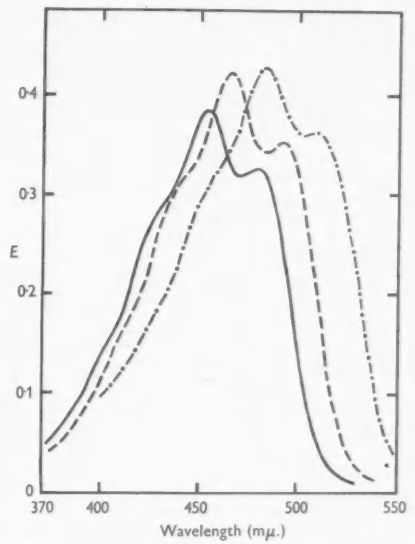

Fig. 2. Absorption spectrum of leprotene in various solvents. Ethanol, ——; benzene, – – –; carbon disulphide, •—•.

Table 3. *Absorption spectra maxima of leprotene in various solvents*

Solvent	Absorption maxima (mμ.)
Light petroleum (b.p. 40–60°)	~428, 452, 479
Carbon disulphide	~455, 483, 512
Benzene	~438, 464, 492
Ethanol	~428, 452, 480

~ Denotes inflexion.

The possibility that pigments *B* and *C* were *cis*-isomers of cryptoxanthin and zeaxanthin respectively appears to be ruled out because, although *cis*-isomers of cryptoxanthin and zeaxanthin have the sharpness of their longest waveband reduced, it is never lost completely as occurs with pigments *B* and *C*, in which it appears only as an inflexion. The absorption spectra were, however, not examined for a possible *cis*-peak in the

region 320–340 mμ. (Zechmeister, 1944, 1954). In the case of pigment *C*, the intensity of absorption in the region 350–370 mμ. relative to that at the main maximum is much greater than normally encountered in a carotenoid; the reason for this is obscure, but it occurred in all preparations (see Figs. 3 and 4). Because of the essential single-banded nature of the absorption spectra of *B* and *C*, they may be ketonic carotenoids (Goodwin, 1952*b*). At the moment they do not appear to be identifiable with any previously described carotenoids. Zones *D* and *E* (Table 1) appeared to be traces of oxidative artifacts and were not examined further.

Turian (1953) has reported the presence of γ-carotene in his strain of *Myco. phlei*. Only once out of numerous analyses was γ-carotene detected in our cultures of his strain; on that occasion it was present only in minute traces.

In the majority of cultures of strain Fleole and the strain used by Turian phytofluene was detected in traces. It was identified by its absorption spectrum, its position on a chromatographic column (below β-carotene), its bright green fluoroescence in ultraviolet light, and by a mixed chromatogram with an authentic specimen from *P. blakesleeanus* (Goodwin, 1952*a*). Phytofluene was never observed in strain Crottin.

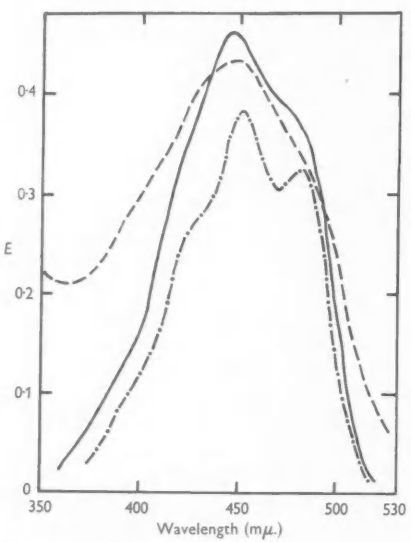

Fig. 3. Absorption spectra in light petroleum of: Pigment *B*, ——; pigment *C*, – – –; zeaxanthin, •—•.

Chrysophlein

Turian (1950*a*, *b*) described chrysophlein as an acidic carotenoid present in his strain of *Myco. phlei*; he adopted the following procedure to obtain this pigment:

> The dried bacteria were extracted with acetone, diluted 1:3 with 2·5% (?w/v) aqueous KOH and shaken with light petroleum, which removed carotenes and esterified xanthophylls. The residual pigment, chrysophlein, was then extracted from the aqueous layer with ethyl ether or, after acidification, with light petroleum. Chrysophlein was considered an acidic carotenoid on the assumption that in the presence of KOH it formed a salt which was soluble in ethyl ether but insoluble in light petroleum; acidification liberated the free pigment which was soluble in light petroleum as well as in ethyl ether.

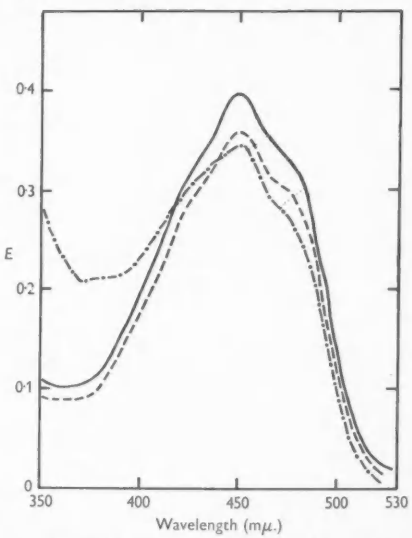

Fig. 4. Absorption spectrum in light petroleum of 'chrysophlein', ——; fraction *A* from chrysophlein (compare pigment *B*, Fig. 3), – – –; fraction *B* from chrysophlein (compare pigment *C*, Fig. 3), •—•.

Attempts have been made to identify chrysophlein with one of the pigments, just described, which were isolated from *Myco. phlei* by more conventional methods. By following exactly the instructions of Turian (1950*b*), specimens of chrysophlein were obtained from Turian's strain and chromatographed on deactivated alumina. Two fractions were separated: the first (*A*) moved off the column with 20% (v/v) ethyl ether in light petroleum and the second (*B*) moved off when the concentration of ether was raised to 50% (v/v). The absorption spectra of the two fractions

Table 4. *Separation of polyenes of fraction A Table* 1 *from diphenylamine cultures of the three strains of* Mycobacterium phlei

Adsorbent, activated + deactivated alumina (4:1); developer, light petroleum containing varying amounts of ethyl ether; polyenes in order of increasing adsorptive power.

Zone	Description	Absorption maxima in light petroleum (mμ.)	Concn. of ether (%, v/v) required for elution	Provisional identification
A	Colourless, no fluorescence in u.v. light	282	1–2	Phytoene
B	Colourless, green fluorescence in u.v. light	332, 348, 368	3–5	Phytofluene
C	Brownish	428, 450, 475	5	β-Carotene
D	Light yellow	424, 456	10	Pigment X*
E	Lemon-yellow	380, 404, 424	10	ζ-Carotene
F	Khaki	441·5, 470	15	Neurosporene
G	Brown	429, 452, 478	15–20	Leprotene
H	Red	445, 470, 501	20–25	Lycopene

* This pigment (Goodwin & Osman, 1953) is reminiscent of flavacene (Tischer, 1938).

Table 5. *Quantitative pigment distribution in the various strains of* Mycobacterium phlei

7-day cultures on standard solid medium; temp. 37°.

	Strain					
	Fleole		Crottin		Turian	
Pigment	Concn. (μg./g. dry wt.)	% of total pigments	Concn. (μg./g. dry wt.)	% of total pigments	Concn. (μg./g. dry wt.)	% of total pigments
Phytofluene	Trace	—	0	—	Trace	—
β-Carotene	12·7	8·7	5·9	9·6	5·5	5·2
ζ-Carotene	Trace	—	0*	—	Trace	—
Leprotene	82·5	57·3	28·4	46·5	51·8	49·3
Lycopene	12·7	8·9	3·0	4·9	1·8	1·7
Pigment *B*	28·2	19·6	8·8	14·6	27·6	26·3
Pigment *C*	7·9†	5·5	14·9†	24·4	18·3†	17·5
Total	144		61		106	

* Occasionally detected in traces.
† These values represent upper limits because of the possible presence of oxidative artifacts.

are given in Fig. 4. It is clear that these pigments are identical with pigments *B* and *C* isolated from the bacteria by the conventional methods (see Table 1 and Fig. 3); this was confirmed by carrying out mixed chromatograms of the chrysophlein fractions with pigments *B* and *C*. Turian also found his chrysophlein separated into two fractions, which he considered to be the free pigment and its salt. Acidic carotenoids, e.g. astacin, would, however, have remained in the saponifiable portion of our extract, whilst *B* and *C* were found in the unsaponifiable portion (Table 1), being extracted along with the neutral carotenoids, β-carotene, leprotene, etc. It is difficult to accept the idea that the potassium salt of chrysophlein is insoluble in light petroleum but soluble in ethyl ether; potassium salts of fatty acids and acidic carotenoids are completely insoluble in ethyl ether. The observations of Turian (1950*a*), which we have confirmed, that under certain conditions chrysophlein is extracted from an aqueous medium by ethyl ether but not by light petroleum may eventually be explained on grounds of partition.

Action of diphenylamine

Chromatography of the mixture of pigments produced when the three strains were grown on the standard solid medium containing diphenylamine (1:35 000) indicated the synthesis of polyenes additional to those present in normal cultures. The separations obtained were the same in all cases (Table 4). The additional polyenes identified were those already found in diphenylamine cultures of *P. blakesleeanus* (Goodwin, 1952*a*) and *Rhodospirillum rubrum* (Goodwin & Osman, 1954), namely phytoene (in traces), phytofluene, ζ-carotene, neurosporene and pigment X. The latter is reminiscent of flavacene (Goodwin & Osman, 1953; Turian & Haxo, 1954), first obtained from the blue-green alga *Aphanomenizon flos-aquae* (Tischer, 1938), but their identity has not yet been demonstrated.

Quantitative distribution of pigments

The amounts of the various components produced on the standard solid medium under the same experimental conditions by the three strains

Table 6. *Relative amounts of polyenes synthesized by diphenylamine cultures of three strains of* Mycobacterium phlei

Standard solid medium + diphenylamine (1:35 000); 7-day cultures; temp. 37°.

	Percentage of total pigments present		
	Fleole	Crottin	Turian
Phytofluene	18·7	5·9	9·5
β-Carotene	2·8	0·8	2·3
Pigment X	16·8	3·4	6·7
ζ-Carotene	3·5	1·4	1·0
Neurosporene	Trace	0	4·4
Leprotene	34·0	23·6	24·1
Lycopene	6·4	15·5	5·4
Strongly adsorbed carotenoids (pigments *B* and *C*)	17·8	49·4	46·6

Table 7. *Effect of diphenylamine and phenol on growth and carotenogenesis in* Mycobacterium phlei

Strain Turian; standard liquid medium; amounts produced in 100 ml. medium in 250 ml. Erlenmeyer flasks; temp. 37°; each value represents the mean of two flasks.

	6-day cultures			10-day cultures		
		Carotenoids			Carotenoids	
Inhibitor	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
A None (control)	358	8·4	23	355	12·5	35
B Diphenylamine (1:35 000)	218	2·6	11	224	5·7	23
C B + $FeSO_4$ (1 mg./100 ml.)	250	2·9	12	216	4·8	22
D Phenol (1:2500)	53	0·6	12	102	2·4	21
E D + $FeSO_4$ (1 mg./100 ml.)	55	0·75	14	103	2·4	21

are recorded in Table 5. It will be seen that, although the distribution of the component pigments is essentially the same in all strains, strain Crottin produced less total pigment than do the other two strains; this is quite obvious visually, the Crottin strain being yellow and the others orange-red. The same pigments are produced in the same amounts when glucose is replaced as the carbon source by glycerol, or when the solid medium is replaced by the standard liquid medium.

In the presence of diphenylamine, the relative amounts of the more unsaturated polyenes are reduced in all strains because of the appearance of the more saturated polyenes (Table 6). The total amounts present were determined only in the case of Turian's strain (Table 7), when considerable reduction was demonstrated. In the other strains the amounts were also obviously reduced because the colour of the diphenylamine cultures of Fleole were yellow-orange and of Crottin pale yellow compared with the deep orange-red and deep yellow of the normal cultures.

In agreement with Turian's observations (1951), phenol was also found to inhibit carotenogenesis in his strain to a greater extent than growth. Phenol inhibition differs, however, from diphenylamine inhibition in that chromatography of the extracts showed that reduction of the synthesis of leprotene, etc., was not accompanied by an augmented synthesis of the more saturated polyenes (phytofluene, etc.). Contrary to the observations of Turian, however, it was not possible to overcome the phenol and diphenylamine inhibitions by Fe^{2+} (Table 7).

SUMMARY

1. Three strains of *Mycobacterium phlei* produce the same carotenoids in the same relative amounts; they are β-carotene, ζ-carotene, leprotene, lycopene and two unidentifiable xanthophylls (*B* and *C*); these last are the pigments previously considered to be cryptoxanthin and zeaxanthin. Turian's chrysophlein appears to be a mixture of the xanthophylls *B* and *C*.

2. The relative amounts of the pigments present in each strain have been determined.

3. Diphenylamine stimulates the synthesis of the more saturated polyenes (phytofluene, etc.), whilst inhibiting that of the more unsaturated polyene (β-carotene, leprotene, etc.). Phenol inhibits synthesis of β-carotene, etc., but does not stimulate synthesis of the phytofluene series. Fe^{2+} does not reverse the effect of diphenylamine or phenol.

We wish to thank the Medical Research Council for a grant towards Laboratory Expenses.

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Studies in Carotenogenesis

18. CAROTENOID PRODUCTION BY A STRONGLY CHROMOGENIC BACTERIUM ISOLATED FROM BUTTER

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(*Received* 20 *April* 1955)

The nature of the carotenoid pigments and the factors controlling their production have been investigated in a strongly chromogenic bacterium isolated from butter by Dr A. Lutz (Strasbourg). It was originally designated *Mycobacterium phlei* strain Pelligrino, but Dr Ruth Gordon (Rutgers University, N.J.), who recently kindly examined the strain, reported that it is not a typical *Myco. phlei*. 'It is not acid-fast but resembles strains coming to us as *Nocardia* spp.' As Topley & Wilson (see Wilson & Miles, 1946) do not recognize the genus *Nocardia*, and as Dr Gordon is 'inclined to agree with them', the difficult taxonomic problem of its complete identification remains unsolved and there does not appear any immediate possibility of its solution. A culture of this organism has been lodged with the DSIR National Collection of Industrial Bacteria, Teddington, Middlesex.

In spite of this difficulty of identification, the results of our investigation are now presented mainly because of their relevance to the general problem of carotenogenesis, but also because the information may eventually help to identify the organism.

EXPERIMENTAL

Culture. The culture of the unidentified organism was kindly supplied by Dr A. Lutz, Strasbourg. In this paper it will be called Pelligrino.

Culture conditions and media. The standard solid medium and standard liquid medium used were those described in the previous paper (Goodwin & Jamikorn, 1956). All media were normally sterilized by autoclaving (15 lb./sq.in./15 min.), although the iron salts used in an attempt to reverse the effect of diphenylamine were sterilized by filtration. Washed suspensions were prepared as described previously for *Rhodospirillum rubrum* (Goodwin & Osman, 1954).

Analytical methods. The methods used for determination of dry weight lipid and total carotenoids and for chromatographically separating and identifying the pigments were those described in the previous paper (Goodwin & Jamikorn, 1956).

RESULTS

Pigments present

Pigment extracts from Pelligrino cultured on the standard solid medium for 10 days were first chromatographed on deactivated alumina. Fraction *A* ran straight through the column and was

Table 1. *First separation of carotenoids of Pelligrino*

Adsorbent, deactivated alumina; developer, light petroleum containing varying amounts of diethyl ether; zones in order of increasing adsorptive power.

Zone	Description	Absorption maxima (mμ.) (light petroleum)	Concn. (v/v) of ether required to elute zone	Provisional identification
A	Yellowish, passes straight through to column	—	0	Mixture
B	Yellowish pink	448*	10–20	Pigments *B* and *C* of *Myco. phlei* (Goodwin & Jamikorn, 1956)
C	Brownish yellow	452*	60–80	
D	Orange-red	439, 458, 488	Ether + 5% ethanol	(?) Dihydroxy-carotenoid, similar to that in *Bacillus lombardo pelligrini* (Chargaff & Lederer, 1935)

* Only one main band.

Table 2. *Separation of Fraction A* (*Table* 1) *of polyenes of Pelligrino*

Adsorbent, activated + deactivated alumina (4:1); developer, light petroleum containing varying amounts of diethy ether; zones in order of increasing adsorptive power.

Zone	Description	Absorption maxima (mμ.) in light petroleum	Concn. (v/v) of diethyl ether required to elute zone	Probable identification
A_1	Colourless, trace of green fluorescence in u.v. light	—	3	Traces of phytofluene
A_2	Orange-yellow	~425, 450, 475	5	β-Carotene
A_3	Yellow-brown	~428, 452, 479	10	Leprotene
A_4	Trace of pink	435, 461, 490	15–20	γ-Carotene

~ Denotes inflexion.

collected in the filtrate. Development of the chromatogram with light petroleum (light petroleum b.p. 40–60° was used throughout this investigation) containing varying amounts of ethyl ether resulted in a chromatogram described in Table 1. Fraction *A* was rechromatographed on a 4:1 mixture of activated and deactivated alumina and a typical separation is given in Table 2. A_1, A_2 and A_3 were identified as phytofluene, β-carotene, and leprotene respectively. They had the correct adsorptive and spectral properties and did not separate on mixed chromatography with specimens of leprotene obtained from various strains of *Myco. phlei* (Goodwin & Jamikorn, 1956). Fraction A_4, which occurs only in traces, has the very characteristic spectrum of γ-carotene. This, together with the fact that its adsorptive power is very similar to that of γ-carotene, makes it reasonably certain that it is this rather characteristic pigment.

Pigments *B* and *C* (Table 1) appear to be identical with those previously observed in three strains of *Myco. phlei* (Goodwin & Jamikorn, 1956). They have similar adsorptive and spectral properties and cannot be separated on mixed chromatography. The concentration of ether required to elute *C* from a column can vary con-

Fig. 1. Absorption spectrum of pigment *D*: in light petroleum, ——; in benzene, – – –.

Table 3. *Separation of hydrocarbon polyenes from diphenylamine cultures of Pelligrino*

Adsorbent, activated + deactivated alumina (4:1); developer, light petroleum containing varying amounts of ethyl ether; polyenes in order of increasing adsorptive power.

Zone	Description	Absorption maxima (mμ.) in light petroleum	Concn. of diethyl ether (%, v/v) required for elution	Identification
A	Colourless; non-fluorescent in u.v. light	282	1–2	Phytoene
B	Colourless, green fluorescence in u.v. light	332, 348, 368	3–5	Phytofluene
C	Brownish	428, 450, 475	5	β-Carotene
D	Lemon-yellow	380, 400, 421	10	ζ-Carotene
E	Yellow	424, 456	10	Pigment X*
F	Khaki	441·5, 470	15	Neurosporene
G	Brown	429, 452, 478	15–20	Leprotene

* (Goodwin & Osman, 1953*b*).

Table 4. *Relative and absolute amounts of constituent polyenes of Pelligrino in presence and absence of diphenylamine*

7-day cultures; standard solid medium.

	Normal cultures		Diphenylamine cultures	
Polyene	Concn. (μg./g. dry wt.)	% of total pigments	Concn. (μg./g. dry wt.)	% of total pigments
Phytofluene	Trace	—	31	10·8
β-Carotene	7·5	2·2	Trace	—
ζ-Carotene	0	0	3	1·1
Pigment X	0	0	34	11·9
Neurosporene	0	0	7	2·4
γ-Carotene	3·7	1·1	3	1·1
Leprotene	110·2	32·4	85	29·5
Pigment *B*	129·5	38·1		
Pigment *C*	55·1	16·2	123	43·0
Pigment *D*	34·0	10·0		
Total	340		286	

siderably from experiment to experiment [compare Table 1 with Table 1 in the previous paper (Goodwin & Jamikorn, 1956); this is often the case with compounds requiring a considerable proportion of ether for elution]. Pigment *D*, which has not been observed in the *Myco. phlei* strains examined, has the adsorptive power of a dihydroxycarotene and an absorption spectrum (Fig. 1) very similar to that of γ-carotene. It may, therefore, be a dihydroxy-γ-carotene. It appears to be very similar to the xanthophyll obtained from *Bacillus lombardopelligrini* by Chargaff & Lederer (1935).

In diphenylamine cultures of Pelligrino, the synthesis of the more unsaturated polyenes (β-carotene, etc.) is inhibited, whilst that of the more saturated polyenes is stimulated. The usual members of these latter series have been identified (Table 3) with those obtained from diphenylamine cultures of *P. blakesleeanus* (Goodwin, 1952). A typical quantitative distribution of pigments in normal and diphenylamine cultures is given in Table 4. It will be noted that both the relative and absolute amounts of the unsaturated polyenes, especially the more strongly adsorbed components, are reduced in the presence of diphenylamine.

Quantitative experiments on liquid media

Reproducibility of results. Table 5 indicated the adequate degree of reproducibility with replicate liquid cultures of Pelligrino under our standard conditions. The concentration of pigments can, however, vary to a greater degree from one experiment to another.

Table 5. *Reproducibility of determination of dry weight and carotenoids in Pelligrino*

Standard liquid medium; amounts per 50 ml. of medium in 150 ml. Erlenmeyer flasks; 8-day cultures; temp. 37°.

		Total carotenoids	
Flask no.	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
1	321	16·0	49·6
2	319	15·3	47·9
3	321	15·7	49·0
4	303	14·8	48·9

Glucose concentration and carotenogenesis. Table 6 shows that growth increases with increasing glucose concentration up to the highest examined (8%, w/v); carotenogenesis, on the other hand, ceases to increase to any great extent above a glucose concentration of 1% (w/v). This results in a lowered concentration of pigments in cultures grown in the presence of high concentrations of glucose. These effects are observed with both 6- and 10-day cultures. This is rather different from that observed with *P. blakesleeanus* (Garton, Goodwin & Lijinsky, 1951), where growth ceases at a glucose concentration of 1% (w/v) whilst β-carotene synthesis continues with concentrations of glucose up to 2·0–2·5% (w/v). It will also be seen that, with the exception of the 0·5% (w/v) glucose medium, where the glucose is probably exhausted after 6 days carotenoid synthesis is relatively greater in old than in young cultures; this is similar to that observed with *P. blakesleeanus* (Goodwin & Willmer, 1952) and *Rsp. rubrum* (Goodwin & Osman, 1953*a*).

Table 6. *Effect of glucose concentration on growth and carotenogenesis in Pelligrino*

Amounts produced in 50 ml. of medium in 150 ml. Erlenmeyer flasks; standard liquid medium containing varying amounts of glucose. Temp. 37°; each value represents the mean of two flasks.

	6-day cultures			10-day cultures		
		Carotenoids			Carotenoids	
Concn. of glucose (%, w/v)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
0·5	138·2	11·6	84	154·2	13·4	87
1·0	192·9	12·0	62	247·4	24·7	99
2·0	238·9	12·2	51	315·5	24·2	77
4·0	286·9	13·4	47	418·7	27·7	66
8·0	333·5	14·2	43	563·5	29·9	53

Table 7. *Growth and carotenogenesis in Pelligrino in presence of glucose or glycerol*

Amounts produced in 50 ml. of medium in 150 ml. Erlenmeyer flasks; standard liquid medium with either glucose or glycerol as carbon source. Temp. 37°; each value represents the mean of two flasks.

	Glucose (2%, w/v) medium			Glycerol (2%, w/v) medium		
		Carotenoids			Carotenoids	
Age of culture (days)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
2	68	1·0	15	18	1·0	56
4	188	5·0	27	49	3·0	61
6	261	11·1	48	82	5·9	72
8	308	19·0	62	106	10·4	98
10	333	23·2	70	120	11·9	99

Effect of glycerol. In the case of Pelligrino replacement of glucose by glycerol in the standard liquid medium results in a considerable reduction of growth, accompanied by a smaller decrease in carotenogenesis (Table 7); there is, therefore, a higher concentration of carotenoids in the glycerol cultures. This agrees with the observations of Ingraham & Steenbock (1935) on *Myco. phlei*. Table 7 also shows that maximal carotenoid production is not reached until the culture is 8–10 days old. Growth is also generally completed by 10 days, but occasionally increases in dry weight have been observed up to 14 days.

Effect of acetate. The effect on carotenogenesis of the replacement of part of the glucose in the standard liquid medium by acetate has been investigated because of the claim of Schopfer & Grob (1950, 1952) that acetate is the primary precursor of carotene in *P. blakesleeanus* and *Mucor hiemalis*. Under our conditions no stimulatory effect of acetate could be demonstrated but both growth and carotenogenesis were reduced to about one-third when acetate completely replaced glucose (Table 8). These observations are in marked contrast to the statement of Barbier & Lederer (1953) that increased growth and lipid production was obtained with *Myco. phlei* by the addition of acetate to Sauton's medium. *iso*Propanol, which Ingraham & Steenbock (1935) found to stimulate pigment synthesis in *Myco. phlei*, reduces it somewhat in Pelligrino (Table 8).

Effect of different nitrogen sources. The substitution of asparagine in the standard liquid medium by either $(NH_4)_2SO_4$ or NH_4NO_3 results in a reduction of growth in both cases (Table 9); carotenogenesis is reduced similarly on $(NH_4)_2SO_4$; but, in contrast, it is reduced to a considerably greater extent on NH_4NO_3; this results in a much lower concentration of carotenoid on the latter medium; on this medium, however, the cells were much more pigmented than usual, being bright red. It was not possible using usual carotenoid solvents to extract most of the red pigments present, indicating that one component was, in all probability, not a carotenoid. Further attempts to extract this residual pigment using hot or cold water or dilute acid or alkali were unsuccessful.

Effect of other inhibitors. The following well-known inhibitors were without any obvious effect on either growth or carotenogenesis: malonate (0·005%, w/v), fluoroacetate (0·001%, w/v), arsenite (0·08M), iodoacetate (0·01%, w/v). Phenol (1:2500) also has no effect, although it is inhibitory to Turian's strain of *Myco. phlei* (Turian, 1950*b*; Goodwin & Jamikorn, 1956). 2:4-Dinitrophenol inhibited both growth and carotenogenesis equally by about 50%; in *Myco. phlei*, 2:4-dinitrophenol stimulates carotenogenesis, according to Turian (1950*b*). Complete inhibition of growth was previous observations with *P. blakesleeanus* (Goodwin *et al.* 1953) and *Rsp. rubrum* (Goodwin & Osman, 1954), but contrary to those of Turian (1950*a*) using his strain of *Myco. phlei*.

Table 8. *Effect of acetate and isopropanol on growth and carotenogenesis by Pelligrino*

Amounts produced in 50 ml. of medium in 150 ml. Erlenmeyer flasks. Standard liquid medium with changes indicated below; each value represents the mean of two flasks.

	6-day cultures			10-day cultures		
		Carotenoids			Carotenoids	
Medium	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
Standard (glucose 2%, w/v)	237	12·4	52	316	27·5	87
Glucose (1%, w/v) + acetate (1%, w/v)	169	10·9	64	204	14·8	73
Acetate (2%, w/v)	108	6·5	60	112	7·6	67
Glucose (2%, w/v) + *iso*-propanol (1%, w/v)	218	9·1	42	249	11·9	48

Table 9. *Effect of altering the nitrogen source on growth and carotenogenesis by Pelligrino*

Amounts produced in 50 ml. of medium in 150 ml. Erlenmeyer flasks; standard liquid medium with different N sources. Temp. 37°; each value represents the mean of two flasks.

	6-day cultures			10-day cultures		
		Carotenoids			Carotenoids	
Nitrogen source (all 0·5%, w/v)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
Asparagine (control)	293	13·7	46	533	36·3	68
$(NH_4)_2SO_4$	226	11·4	50	333	23·3	69
NH_4NO_3	158	3·8	29	236	4·6	20

Attempts to reverse the effects of diphenylamine. Neither adenosine 5′-phosphate (AMP) nor riboflavin, both of which reverse the inhibitory action of diphenylamine on carotenogenesis in *P. blakesleeanus* (Goodwin, Jamikorn & Willmer, 1953), has any marked effect in Pelligrino. Riboflavin at a concentration of 1:5000 completely inhibits growth of the organism. Furthermore, it was not possible to reverse the diphenylamine effect with either Fe^{2+} or Fe^{3+}. This is in agreement with our observed with 0·01% (w/v) fluoride, 0·02% (w/v) iodoacetate and 0·001% (w/v) streptomycin; lower concentrations of streptomycin tend to inhibit carotenogenesis to a greater extent than growth. This is similar to the observations made with *Rsp. rubrum* (Goodwin & Osman, 1953*a*), but different from the very marked effect observed with *P. blakesleeanus* (Goodwin, Griffiths & Modi, 1956) and *Euglena gracilis* var. *bacillaris* (Goodwin & Jamikorn, 1954).

Effect of Tween 80. In preliminary experiments designed to examine the role of fat-soluble substances in carotenogenesis, the effect of the dispersing agent (Tween 80) was examined as a control. It was found that Tween 80 (0·02%, w/v) completely inhibited growth in Pelligrino. This inhibition was completely overcome when a fatty substance such as squalene or liquid paraffin (0·1%, w/v) was dispersed in the medium with the Tween. Furthermore, under these conditions it was found that squalene had no demonstrable effect on the biosynthesis of carotenoids in this organism.

Table 10. *Effect of light and temperature on growth and carotenogenesis by Pelligrino*

Amounts produced in 50 ml. of medium in 150 ml. Erlenmeyer flasks; 'Light' cultures were incubated in a thermostatically controlled water bath illuminated at a distance of 10–12 in. by two 80 w daylight fluorescent lamps. Each value represents the mean of two flasks.

	6 days			10 days		
		Carotenoid			Carotenoid	
Temp. of incubation	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)	Total dry wt. (mg.)	Amount (μg.)	Concn. (p.p.m.)
			Light cultures			
15°	38	1·7	45	51	2·8	55
32°	161	9·1	56·5	246	16·8	68
37°	—	—	—	—	—	—
			Dark cultures			
15°	35	1·8	51	101	5·5	54
32°	171	10·3	60	277	19·3	70
37°	249	16·2	65	327	23·3	71

Effect of light and temperature. Table 10 shows that light has no effect on carotenogenesis either at 15° or 32°. Growth is greater at the higher temperature after 6 and 10 days and carotene concentration is also somewhat increased. Pelligrino thus resembles *P. blakesleeanus* in producing less carotenoids at lower temperatures (Friend & Goodwin, 1954), but differs in not being affected by light (Garton *et al.* 1951).

Effect of pH. Growth of Pelligrino on the standard liquid medium was obtained only over the narrow pH range 6·0–7·0. There appeared to be slight variations in growth rate after 6 days on media with pH values of 6·0, 6·5 and 7·0, but after 10 days there were no significant differences between these media. In no case was there any significant differences in carotenogenesis.

Experiments with washed suspensions

Six-day cultures grown on the standard medium, either alone or in the presence of diphenylamine, were washed free from original medium and resuspended in the same volume of a medium consisting either of phosphate buffer (M/15, K_2HPO_4, KH_2PO_4) or phosphate buffer plus glucose (2·0%, w/v) and incubated for 24 hr. A large number of experiments were carried out but no significant additional carotenoid synthesis was ever observed even in resuspended diphenylamine cultures. Studies with labelled acetate, however, show that there is a turnover of pigments in washed suspensions (E. Fisher, personal communication).

DISCUSSION

Apart from the relevance of these pigment studies to the general problem of carotenogenesis which has been discussed briefly in the 'results' section, the question arises whether they can help the bacterial taxonomist in assigning the correct classification to this bacterium. Pelligrino synthesizes all the pigments found in 'true' strains of *Myco. phlei* except lycopene (Goodwin & Jamikorn, 1956) and synthesizes a further xanthophyll, pigment *D*. These two differences, absence of lycopene and presence of *D*, cannot, however, be taken as a sufficient differentiation, because strain Lombardo Pelligrini which is acid-fast, produces the same pigments as Pelligrino (Chargaff & Lederer, 1935); furthermore, Pelligrino produces leprotene which has not yet been observed outside the Mycobacteriaceae. It was suggested by Dr Lutz that 'Pelligrino' and 'Lombardo Pelligrini' are the same strain. The only marked difference is that Pelligrino is not acid-fast. As acid-fastness is not an immutable property and in some strains is particularly liable to disappear on continual subculturing under laboratory conditions (A. W. Downie, personal communication), the possibility remains that these two strains are basically the same. This conclusion would be supported by the identity of pigmentation. It will be interesting to see whether bacteriologists will eventually be able to decide unequivocally that Pelligrino is not a species of *Myco. phlei*.

SUMMARY

1. Pelligrino synthesizes phytofluene, β-, ζ- and γ-carotenes, leprotene, and three unknown xanthophylls, two of which also appear in various strains of *Mycobacterium phlei*.

2. Diphenylamine inhibits the synthesis of the more unsaturated polyenes but stimulates the production of phytoene, phytofluene, ζ-carotene, neurosporene and pigment X. The diphenylamine effect is not overcome by riboflavin, adenosine-5′-phosphate, Fe^{2+}, or Fe^{3+}.

3. Maximal carotenoid concentration occurs about 10 days after inoculation and with a glucose concentration of about 1% (w/v). Higher glucose levels increase growth but not carotenogenesis. Replacement of glucose by glycerol reduces growth and enhances carotenogenesis. Acetate reduces growth but has little effect on pigmentation.

4. Replacement of asparagine by $(NH_4)_2SO_4$ reduces growth and carotenogenesis equally, whilst NH_4NO_3 inhibits carotenogenesis much more than growth.

5. Concentrations of streptomycin less than 0·001% (w/v) tend to inhibit carotenogenesis to a greater extent than growth, whilst a number of common metabolic inhibitors when they did not inhibit growth completely did not have a preferential effect on carotenogenesis.

6. Tween 80 (0·02%, w/v) completely inhibits growth of Pelligrino; this is overcome when a lipid-soluble material (squalene or liquid paraffin, 0·1%, w/v) is dispersed in the medium with Tween.

7. Washed suspensions of Pelligrino did not synthesize additional amounts of carotenoids.

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The Metabolism of Pteroylglutamic Acid by the Rat

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From evidence published so far it appears that before pteroylglutamic acid (PGA)* can carry out its metabolic functions the organism must first convert it into the tetrahydro form and that citrovorum factor (CF) may represent one of the metabolically active forms of folic acid (FA). Evidence has been presented (Nichol, 1950; Doctor *et al.* 1953; Doctor, Elam, Sparks, Lyman & Couch, 1954) for the existence in liver of an enzyme that is involved in the conversion of PGA into CF. Moreover, Nichol (1953*a*) has demonstrated that the liver is the main site of this conversion in the body. Of particular interest is the finding of Welch, Nichol, Anker & Boehne (1951) and of Broquist, Stokstad & Jukes (1951) that more CF is formed

* As used by Welch & Nichol (1952) the term 'pteroylglutamic acid' has been restricted to the synthetic compound and '*citrovorum factor*' to the substance(s) promoting growth of *Pediococcus cerevisiae* 8081 (*Leuconostoc citrovorum*); the term 'folic acid' has been considered a generic term applicable to substances which stimulate the growth of *Streptococcus faecalis*. The term 'unconverted folic acid' relates to the substance(s) enhancing the growth of *Strep. faecalis* but not of *Pediococcus cerevisiae*.

when ascorbic acid is present. Since injection of ascorbic acid increases the urinary excretion of CF, it would appear that ascorbic acid brings about favourable reducing conditions essential for the formation of CF.

Since the conversion of PGA into CF by rat and chicken livers has hitherto been studied under normal nutritional conditions only, the question arose whether lack of nutrients or an unbalanced diet would impair this reaction. Before approaching this problem experimentally it was necessary to study the normal capacity of the liver to convert PGA into CF under adequate nutritional conditions. The first part of our study presents the urinary excretion and liver concentration of FA and CF of normal rats after treatment with varying amounts of PGA and ascorbic acid. In the second part liver FA and CF were determined in rats which were kept on diets of various fat and protein contents.

METHODS

Young male rats weighing 40–50 g. were used throughout. The diets employed are presented in Table 1.

For production of choline deficiency, choline chloride was omitted from the low-protein, high-fat diet. The presence of choline deficiency was verified by determining the accumulation of fat in the liver (average: 14·8% in deficient rats, 5·7% in the livers of control rats).

FA and CF in urine and liver were determined with *Strep. faecalis* (Association of Vitamin Chemists, 1951) and *Pediococcus cerevisiae* 8081 (*Ln. citrovorum*) (Sauberlich & Baumann, 1948) respectively. Synthetic CF (Leucovorin, Lederle Laboratories, American Cyanamid Co.) in the form of the hydrated barium salt was used as standard. Since leucovorin is only half as active as the 'natural' CF occurring in horse liver (Keresztesy & Silverman, 1951; Cosulich, Smith & Broquist, 1952), microbiological assay values in terms of leucovorin were divided by two to give results as naturally occurring CF. For determination of FA and CF in liver, these organs were homogenized and autolysed as described by Dietrich, Monson, Gwoh & Elvehjem (1952). Values of 'unconverted FA' were obtained by subtraction of *Strep. faecalis* activity due to CF present from observed values of total *Strep. faecalis* activity. Whilst the work reported here was in progress, the paper by Chang (1953) describing an improved method of liberation of CF in natural materials came to our attention. It was thought advisable, however, not to adopt another method in the midst of our experiments in order to make possible a comparison of all the results.

Liver N was determined by the conventional Kjeldahl method and ascorbic acid with 2:6-dichlorophenolindophenol (Mindlin & Butler, 1938).

RESULTS

Effect of PGA and ascorbic acid on urinary excretion and liver concentration of FA and CF. In the first series of experiments groups of ten to twelve rats were kept for 2 weeks on the control diet During the last 3 days they were injected subcutaneously with different doses of PGA and ascorbic acid. Urine was collected, and at the end of the 3-day period the animals were killed and their livers assayed for FA and CF. The results are shown in Table 2.

It follows from the table that, with increasing doses of PGA, increasing amounts of both FA and CF are excreted in the urine. The upper excretion limit, however, seems to be reached with the administration of 5–10 mg. of PGA/100 g. body weight, since injection of 20 mg. of PGA does not raise the excretion of either FA or CF any further. With each level of PGA treatment excretion of FA surpasses by far (30- to 200-fold) that of CF.

In contrast to urine, the concentration of liver CF is much higher than that of 'unconverted FA' (after subtraction of *Strep. faecalis* activity due to CF). Values in the upper range which lie between 5·3 and 9·8 μg. are already reached with 0·10 mg. of PGA/100 g. body weight, whereas the content of unconverted FA does not exhibit any consistent variation over a wide range of PGA administration. Injection of 5 mg. of PGA/100 g. or more, however, increases significantly the liver concentration of unconverted FA and leads to a marked decrease in the ratio of CF to unconverted FA in the liver. It seems that this is the upper limit of the capacity of the liver to convert PGA into CF efficiently.

Table 1. *Composition of diets*

The diets were supplemented with the following vitamins (mg./100 g. ration): thiamine 0·2, riboflavin 0·3, pyridoxine 0·1, calcium pantothenate 1·6, niacin 5·0 and choline chloride 100. Each rat received 100 i.u. of vitamin A, and 4 i.u. of vitamin D twice weekly

	Diet				
Ingredient	Control	High-fat	Low-protein low-fat	Low-protein high-fat	Protein-free
Casein	18	18	8	8	0
Cornstarch	73	58	83	68	91
Vegetable oil	5	10	5	10	5
Lard	0	10	0	10	0
Salt mixture, U.S.P. 2	4	4	4	4	4

Injections of increasing doses of ascorbic acid, along with a constant dose of PGA (0·25 mg./100 g.), were found to induce increasing excretion of both FA and CF. With 25 mg. of ascorbic acid recovery of more than 90% of the injected dose of PGA was obtained as FA and CF in urine. Equally, treatment with ascorbic acid also raised the liver concentration of both these substances. Whereas no great variations were observed in the CF content, large doses of ascorbic acid seem to raise significantly the concentration of unconverted FA in the liver and thus to lower the ratio of CF to unconverted FA.

these conditions being unable to convert the PGA efficiently into CF stores an increased amount of unconverted FA. The consequence is a fall in the CF/FA quotient, and an apparent impairment of the capacity of the liver to produce CF.

Increased urinary excretion of CF was also observed as a consequence of increased endogenous ascorbic acid production (Table 3). In this experiment eleven rats were treated with sodium phenobarbital, which was incorporated into the control diet at a level of 1·5 g./kg. Rats receiving this drug excreted 3·43 mg. of ascorbic acid (standard error ± 0·32 mg.)/day as against 0·22 ± 0·07 mg.

Table 2. *Urinary excretion and liver concentration of folic acid and citrovorum factor after injection of different doses of pteroylglutamic and ascorbic acids*

Each group comprises 10–12 rats which were kept on a control diet during 2 weeks. During the last 3 days they were given subcutaneous injections of pteroylglutamic and ascorbic acid respectively. The figures in columns 1 and 2 indicate the daily doses per 100 g. body weight. The values for folic acid are the observed values for total folic acid minus *Strep. faecalis* activity due to citrovorum factor present. Figures in columns 3–7 are means ± S.E.M.

Compound injected		Urinary excretion/(μg./100 g. body weight/day)		Liver			
Pteroylglutamic acid (mg.)	Ascorbic acid (mg.)	Folic acid	Citrovorum factor	Wt. (g.)	Folic acid (μg.)	Citrovorum factor (μg.)	Citrovorum factor / Folic acid
—	—	2·7 ± 0·57	0·025 ± 0·004	3·3 ± 0·13	0·55 ± 0·24	3·3 ± 0·53	6·0
0·05	—	17·4 ± 2·68	0·11 ± 0·02	3·3 ± 0·21	0·50 ± 0·56	3·6 ± 0·85	7·2
0·10	—	17·7 ± 2·04	0·10 ± 0·02	3·5 ± 0·13	0·40 ± 0·28	5·8 ± 0·59	14·5
0·25	—	53·8 ± 3·43	0·19 ± 0·13	3·3 ± 0·21	1·15 ± 0·38	6·9 ± 1·45	6·0
0·50	—	102 ± 19·0	0·30 ± 0·07	3·3 ± 0·15	0·40 ± 0·25	5·3 ± 0·79	13·2
1·00	—	163 ± 22·7	0·61 ± 0·08	3·3 ± 0·19	1·00 ± 0·38	8·5 ± 1·36	8·5
2·00	—	267 ± 35·9	1·43 ± 0·31	3·2 ± 0·09	0·54 ± 0·28	6·9 ± 0·83	12·8
5·00	—	421 ± 53·6	2·44 ± 1·00	3·5 ± 0·16	2·10 ± 0·85	9·8 ± 1·20	4·7
10·00	—	456 ± 48·4	4·17 ± 1·43	2·8 ± 0·11	1·80 ± 0·60	5·3 ± 1·35	2·9
20·00	—	438 ± 30·0	3·03 ± 1·15	2·9 ± 0·16	8·70 ± 1·23	8·1 ± 1·15	0·9
0·25	0·25	130 ± 28·5	0·75 ± 0·20	3·6 ± 0·11	1·90 ± 1·04	13·7 ± 1·47	7·2
0·25	1·00	223 ± 64·2	1·27 ± 0·41	3·6 ± 0·10	2·31 ± 0·96	12·3 ± 1·75	5·3
0·25	5·00	207 ± 64·8	3·97 ± 1·09	3·7 ± 0·13	4·79 ± 1·51	15·5 ± 2·00	3·2
0·25	25·00	228 ± 77·0	3·59 ± 0·68	3·4 ± 0·07	6·80 ± 4·06	10·9 ± 1·63	1·5

Table 3. *Effect of ascorbic acid on urinary folic acid and citrovorum factor*

Folic acid and citrovorum factor excretion is given as mean values ± S.E.M. for 11 rats. Figures for folic acid are corrected for the presence of citrovorum factor (see Table 2).

Treatment	Excretion (μg./100 g. body wt./day)	
	Folic acid	Citrovorum factor
None (control period)	1·16 ± 0·17	0·035 ± 0·009
Animals fed diet containing sodium phenobarbital (1·5 g./kg.)	0·94 ± 0·12	0·153 ± 0·024
Ascorbic acid (5 mg./day) added to urine-collection flasks	0·75 ± 0·15	0·044 ± 0·016

It would appear, therefore, that ascorbic acid protects not only CF from oxidation, leading to its increased concentration in both urine and liver, but has a similar protecting effect on FA, too. Ascorbic acid, given along with a small dose of PGA (0·25 mg./100 g.) leads to an increased PGA content of the liver, which is similar to that following a large dose of PGA (5–10 mg./100 g.) given without ascorbic acid. The liver under both

when untreated. Excretion of CF, too, rose significantly during a 3-day urine-collection period, whereas the FA concentration of the urine remained unchanged. In a further control experiment 5 mg. of ascorbic acid were added daily to the urine-collection flasks, and the rats did not undergo any treatment with either ascorbic acid or phenobarbital. No statistically significant changes of urinary concentration of FA or CF were noted.

'Endogenous' ascorbic acid, i.e. ascorbic acid formed in the body under the stimulation of phenobarbital, appears thus to increase urinary CF excretion as much as 'exogenous' ascorbic acid, whereas the *in vitro* addition of this substance to the collected urine is without any effect.

Effect of diet on the conversion of PGA into CF. In this series of experiments young rats were kept on different diets containing 0, 8 or 18% of casein and 5 or 20% of fat respectively. After 3 weeks on these diets, half of each dietetic group was dosed daily with 0·25 mg. of PGA and 5 mg. of ascorbic acid/100 g. body weight during 3 days. On the fourth day all rats were killed and their livers examined for FA and CF. The results obtained are presented in Table 4.

the capacity of the liver to synthesize CF from PGA, and in severe protein deficiency the ability of the liver to store injected PGA is impaired to a marked degree.

The depression of CF synthesis by rats kept on diets low or deficient in protein is accompanied by a similar decrease in liver nitrogen. Livers of twenty-one rats maintained on the control diet, of thirteen rats on the low-protein diet and of eleven rats on the protein-free diet were examined for nitrogen content. The following amounts (mg./g. of liver) with their standard errors were found: $30{\cdot}8 \pm 0{\cdot}74$; $25{\cdot}9 \pm 1{\cdot}03$; $23{\cdot}9 \pm 0{\cdot}87$. Whereas the livers of rats on the low-protein diet exhibited significantly lower values than those of the controls, the difference between the two latter groups proved

Table 4. *Effect of diet on liver levels of folic acid and citrovorum factor*

Figures in columns 6–8 are means ± S.E.M. Each group comprises 10–12 rats which were kept during 3 weeks on the specified diets. Values for folic acid are corrected for presence of citrovorum factor (see Table 2). A + in column 5 indicates daily subcutaneous injections of 0·25 mg. pteroylglutamic acid (PGA) and 5·0 mg. ascorbic acid (AA)/100 g. body weight during the last 3 days of experiment; – indicates no injection.

	Diet				Liver			
Wt. change (g.)	Casein (%)	Fat (%)	Choline (%)	PGA + AA	Wt. (g.)	Folic acid (μg.)	Citrovorum factor (μg.)	Citrovorum factor / Folic acid
+43	18	20	0·01	–	4·4 ± 0·27	0·35 ± 0·21	3·07 ± 0·07	8·8
+48	18	20	0·01	+	4·6 ± 0·20	2·43 ± 1·47	7·79 ± 1·97	3·2
+28	8	5	0·01	–	3·1 ± 0·21	0·50 ± 0·24	0·47 ± 0·15	0·9
+27	8	5	0·01	+	3·4 ± 0·15	2·45 ± 0·63	1·41 ± 0·44	0·6
+20	8	20	0·01	–	2·9 ± 0·25	0·40 ± 0·51	0·19 ± 0·12	0·5
+23	8	20	0·01	+	3·1 ± 0·15	2·51 ± 0·75	1·14 ± 0·50	0·5
+21	8	20	0	–	3·9 ± 0·21	0·66 ± 0·17	0·34 ± 0·10	0·5
+20	8	20	0	+	3·7 ± 0·32	2·38 ± 1·58	1·02 ± 0·33	0·4
–13	0	5	0·01	–	1·4 ± 0·05	0·40 ± 0·08	0·09 ± 0·02	0·2
–12	0	5	0·01	+	1·4 ± 0·05	0·63 ± 0·10	0·24 ± 0·04	0·4

As can be seen from Table 4, a moderately high fat diet with a sufficient protein content (18%) does not significantly affect the FA and CF content of the liver. Similar values for FA, CF and CF/FA quotients were obtained as with a low-fat diet (Table 2). Reduction of the protein content of the diet from 18 to 8%, however, significantly depressed the CF content of the liver, whereas the level of unconverted FA was not affected. Very low values of the quotient CF/unconverted FA were therefore noted. On the other hand, neither the fat content of the diet nor an accompanying choline deficiency had any influence on the FA and CF levels of the liver under these conditions. Rats maintained on a protein-free diet had a considerably lower liver level of CF than those on the low-protein diet, and, when treated with PGA, were not able to store unconverted FA in their livers. The quotient CF/unconverted FA was found to be lower than in livers of rats on low-protein diets. It seems, therefore, that lack of dietary protein lowers to be statistically insignificant. Rats maintained on the low-protein diet, while growing much better than rats on the protein-free diet, have a similarly low level of liver nitrogen and impaired capacity to synthesize CF.

DISCUSSION

The experimental results presented above demonstrate that PGA, when introduced parenterally into the body, is to a large part excreted with the urine as FA. The recovery of the injected dose, however, decreases from about 30% when 0·05 mg./100 g. body weight is given to less than 2% with a 20 mg. dose. It is noteworthy that CF accounts for 0·5–3% only of the total FA excreted in urine. Thus the percentage of the dose of PGA recovered in urine as CF decreases from 0·2 after injection of 0·05 mg./100 g. body weight to not more than 0·015 when 20 mg. of PGA are administered. On the other hand, the absolute amount of CF

excreted increases more than 100-fold when large doses of PGA are given. These figures show the limited ability of the organism to convert injected PGA into CF.

In contrast to urine most of the FA found in liver proved to be CF, and only 6–13 % was unconverted FA. Treatment with PGA increased the liver content of CF by not more than a few micrograms; relatively very large doses of PGA, 5 mg. or more/100 g. body weight, increased the concentration of unconverted FA as well. Under these conditions the liver is apparently not able to convert PGA into CF efficiently.

Administration of ascorbic acid has been shown to increase CF excretion in urine of man (Welch *et al.* 1951; Broquist *et al.* 1951; Gabuzda, Phillips, Schilling & Davidson, 1952) and rats (Welch *et al.* 1951; Guggenheim, Leibowitz & Rakower, 1953) and to augment CF formation by liver of rats (Nichol, 1950; Broquist *et al.* 1951) and chicken (Doctor *et al.* 1954; Nichol, 1953*b*). The effect of the vitamin seems to be due to its reducing property, since glucoascorbic acid (Welch *et al.* 1951) and *iso*ascorbic acid (Guggenheim *et al.* 1953), as well as cysteine and homocysteine (Doctor & Trunnell, 1954), likewise enhance urinary excretion of CF. In our experiments ascorbic acid increased the excretion not only of CF but also that of FA. When a dose of 0·25 mg. of PGA/100 g. body weight was injected without additional ascorbic acid, 22 % of it was recovered in urine. The recovery rose to 52 %, however, when the same amount of ascorbic acid was injected along with PGA, and even to 91 % when the dose of ascorbic acid was increased 100-fold. Excretion of CF rose concomitantly, and in terms of the administered PGA without ascorbic acid it was 0·08 %. After injection of 0·25 mg. of ascorbic acid/100 g. body weight, the excretion of CF rose to 0·3 % and after injection of 25 mg. of ascorbic acid it rose to 1·4 %.

The increase in liver FA and CF after administration of ascorbic acid is much smaller. Ascorbic acid was found to increase the CF level by 70–125 %, and that of unconverted FA by 70–490 %. It may be assumed that the amount of PGA available to the liver under the influence of large doses of ascorbic acid overtaxes its capacity to convert it into CF, and as a consequence part of it is stored as unconverted FA.

Although variation of the fat content of the diet and even fat accumulation in the liver due to choline deficiency were not found to affect the conversion of FA into CF, protein deficiency had a marked influence. The liver concentration of CF decreases with diminishing protein content of the diet, and in severe protein deficiency the capacity of the liver to store injected PGA is also impaired. Protein deficiency therefore profoundly affects the metabolism of PGA by impairment of its hepatic conversion into CF.

Since the most striking effect of PGA deficiency in man is macrocytic anaemia associated with megaloblastic erythropoiesis, the question arises as to whether the macrocytic anaemia that occurs in children suffering from Kwashiorkor (Walt, Wills & Nightingale, 1950; Brock & Autret, 1952; Joint FAO/WHO Expert Committee on Nutrition, 1953; Autret & Behar, 1954; Adams, 1954) is due to the impaired conversion of PGA into CF as a result of an insufficient intake of dietary protein. The observations of some workers (Pieraerts, 1949; Trowell, Davies & Dean, 1952) that this anaemia responds to protein supplementation of the diet but not to PGA administration are of special interest in the light of our findings reported above.

SUMMARY

1. Young rats when injected with pteroylglutamic acid (PGA) increase the urinary excretion of folic acid (FA) and citrovorum factor (CF), but the recovery decreases with increasing doses. At each excretion level, CF accounts for only 0·5–3 % of the total FA.

2. In liver more CF than unconverted FA is found. Whereas administration of 0·05–2·0 mg. of PGA/100 g. body weight increases the CF content of the liver by only a few micrograms without increasing the concentration of unconverted FA, larger doses (5–20 mg./100 g.) lead to a higher storage of unconverted FA without further increases in the CF level. As a consequence, the ratio of CF to unconverted FA is decreased.

3. Ascorbic acid, when injected together with PGA, increases the urinary excretion of both FA and CF. It raises the liver level of CF and, when injected in larger doses, leads to an increased storage of unconverted FA as well. Under these conditions the ratio of CF to unconverted FA is again diminished.

4. Rats treated with phenobarbital increase their excretion of both ascorbic acid and CF. *In vitro* addition of ascorbic acid to collected urine is without any influence on its CF concentrations.

5. Low-protein and protein-free diets lower the liver content of CF and the ratio of CF to unconverted FA. In severe protein deficiency the ability of the liver to store injected PGA is also impaired. It is concluded that lack of dietary protein depresses the capacity of the liver to convert PGA into CF.

6. Both high-fat diets and fat accumulation in the liver due to choline deficiency do not impair its capacity to form CF.

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Changes in the Composition of the Fat of *Aspergillus nidulans* with Age of the Culture

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While studying linseed oil Ivanow & Klokow (1933) observed that as ripening of the oil-bearing seeds proceeded the proportion of the linolenic acid component increased, whereas those of the oleic and linoleic acid components decreased. From the results of feeding experiments with rats Boxer & Stetten (1944) formed the opinion that in these animals oleic acid was formed from stearic acid by dehydrogenation and that saturated acids are the primary products in fat biosynthesis. Coleman, Cefola & Nord (1952) studied formation of fat in *Fusarium lini* Bolley, and concluded that a mechanism for the dehydrogenation of fatty acids operates in this mould. Such micro-organisms, which can synthesize relatively large quantities of fat when grown on a simple defined medium of sugar and inorganic salts, may prove useful agents for the study of the reactions which give rise to fat, for both substrate and cultural conditions can be well regulated. Recently, we examined the fat of *Aspergillus nidulans* (Singh, Walker & Meara, 1955), and we have now conducted experiments which were planned to throw light on the question whether the unsaturated acids that are components of the fat of this organism are formed by dehydrogenation of saturated acids or by some other mechanism.

EXPERIMENTAL

Aspergillus nidulans Eidam was grown as surface cultures from spores sown on a medium which contained (%, w/v): NH_4NO_3, 0·6; $NaH_2PO_4,2H_2O$, 0·73; K_2SO_4, 0·044; $MgSO_4,7H_2O$, 0·5; $FeCl_3,6H_2O$, 0·016; $ZnSO_4,7H_2O$, 0·005; sucrose, 34; the medium was adjusted to pH 3·8 and made up to volume with distilled water. In each experimental series a set of Pyrex penicillin flasks, each containing 175 ml. of this medium, was employed. After sterilization in steam at 10 lb. pressure for 15 min. the flasks were cooled, their contents inoculated and incubated at 25°. Cultures were harvested after 5, 7, 9, 14 and 21 days' incubation. The number of flasks taken at any given time depended upon the growth attained; thus at 5 days it was necessary to combine the contents of twenty flasks in order to obtain sufficient fat for detailed analysis. At the later periods, a smaller number of flasks yielded sufficient fat for this purpose, but the number of flasks withdrawn at any

time was never less than five. After the cultures had been killed by heating in free steam for 10 min. the mycelial felts were removed from the flasks and separated from adhering medium by washing with distilled water on a Büchner funnel. The filtrates from the flasks and the washings were combined, and after suitable dilution (the total metabolism solution from a set of five flasks was made up with distilled water to 2 l.) the residual sucrose was estimated by the method of Lane & Eynon (1923). The felts were dried in air for 3 or 4 days and then in an air-oven at 60° until the weight was constant. The material so obtained was broken down in a small grinding mill and thoroughly extracted with light petroleum (b.p. 40–60°) in a Soxhlet apparatus. The solvent was distilled from the extracted fat, the last traces of petroleum being removed by heating the residue *in vacuo* for 15 min. at 100°. Estimations of acid value, saponification equivalent, iodine value and unsaponifiable matter were then performed on the fat, the procedures being those recommended by Hilditch (1949). The mixed fatty acids were examined spectrophotometrically for their contents of linoleic and linolenic acids, according to the method of Hilditch, Patel & Riley (1951).

RESULTS

The changes in the weight of felt and in its content of fat with time of incubation are recorded in Table 1. The combined contents of a number of flasks, in no case less than five, were used for each estimation. The results have been calculated on the average contents of one flask and the standard deviation is shown beneath the mean weight of felt. The chemical characteristics of fat samples isolated from felts which were harvested at different stages of the incubation period are given in Table 2. The component fatty acids of the different samples of fat are recorded in Table 3. The weights of the individual fatty acids obtained by saponification of the different samples of fat are given in Table 4. These weights refer in all cases to the contents of one flask.

The rate of synthesis of fat in *A. nidulans* is seen to be slow in the early period of incubation, but it increases rapidly about the seventh day, the maximum weights of felt and fat being attained on the ninth day. Between this stage and the fourteenth day there occurs a slight fall in the weight of the felt and an appreciable fall (approx. 14%) in the weight of fat present in the felt. Thereafter the weight of felt remains almost stationary for the next 7 days, but a further fall occurs in the weight of the fat, to a value about 64% of that attained on the ninth day. The iodine value falls to a minimum on the ninth day and then rises appreciably to its highest value, attained on the twenty-first day. From the proportions of the component acids shown as percentages in Table 3 it is clear that, during the early period of incubation, oleic acid is the predominant component, and at 7 days its ratio to the percentage of total saturated acids is more than 2:1. By the ninth day, however, the percentage of the total saturated acids has risen appreciably and reached its maximum, while that of oleic acid has fallen to a value above which it rises only slightly in the subsequent period of incubation. Also, after the ninth day the percentage of total saturated acids again falls to an

Table 1. *Changes in the weight of the mycelium of* Aspergillus nidulans *and in its content of fat during different periods in the development of the mould in* 175 *ml. of medium. Sucrose present initially,* 59·5 *g.*

Time of incubation (days)	Sucrose utilized (g.)	Dry felt (g.)	Dry felt (g./100 g. of sucrose utilized)	Fat (g.)	Fat (g./100 g. of dry felt)	Fat (g./100 g. of sucrose utilized)
5	20·70	3·956 (±0·463)	19·1	0·585	14·8	2·8
7	28·25	5·803 (±0·782)	20·5	0·892	15·4	3·2
9	43·45	14·116 (±0·514)	32·5	6·310	44·7	14·5
14	49·06	13·557 (±0·442)	27·6	5·395	39·8	11·0
21	54·90	13·636 (±0·371)	24·8	4·080	29·9	7·4

Table 2. *Chemical characteristics of the fat of* Aspergillus nidulans *after different periods of incubation*

Time of incubation (days)	Acid value	Iodine value	Saponification equivalent	Unsaponifiable matter (g./100 g. of fat)
5	2·90	85·5	291·2	8·0
7	1·70	83·8	292·9	5·2
9	0·84	73·7	290·2	1·3
14	0·60	80·8	289·1	1·7
21	1·50	88·3	292·5	2·8

appreciable extent. Table 4, in which are given the actual weights of the component acids, shows clearly that the quantities of all the component acids, namely the saturated ones together with oleic and linoleic acids, increase in the earlier period of incubation up to the point at which the mould's activity reaches its peak. Then, when incubation is prolonged beyond 9 days, the actual weights of all these components decrease, the fall being relatively small in the case of linoleic acid but much greater in the other cases.

In experiments with three species of mould fungus, one of which was *A. nidulans*, Gregory & Woodbine (1953) found that greater unsaturation in the fat was associated with longer incubation. They indicated, however, that this need not imply that the longer period had enabled synthesis of the unsaturated acid(s) to proceed from the already synthesized fatty acids. They quote a private communication from Dr J. A. Lovern to the effect that 'this whole subject of fatty acid production, however, can only be satisfactorily evaluated on a basis of quantitative estimations of particular and individual fatty acids'. The results reported in the present communication provide evidence of the kind indicated by Hilditch and by Lovern as necessary to enable conclusions to be drawn about the question under discussion. It is seen that although the proportions of the component acids of the fat vary during the period of fat synthesis in *A. nidulans*, the actual weight of each component shows at first an increase up to a limit, and then, after the point of maximal synthetic activity has been attained, the actual weight of each acid decreases. The evidence as a whole affords no support for the view that during the biosynthesis of fat the unsaturated acids are formed by dehydrogenation of the saturated acids. This verdict is in agreement with the finding of Bloch (1948), who concluded from the results of experiments made with 'labelled' acetate and pyruvate that saturated and unsaturated acids arise by reactions proceeding along different pathways.

Table 3. *Changes in the composition of the fat of* Aspergillus nidulans *with time of incubation*

Time of incubation (days)	Mixed fatty acids		Component fatty acids as percentages of total component fatty acids			
	Iodine value	$E^{1\%}_{1\,cm.}$ at 234 mμ.	Saturated	Oleic	Linoleic	Linolenic
5	82·2	182	29·1	50·8	20·1	—
7	81·1	147	26·4	57·4	16·2	—
9	73·7	155	35·5	47·4	17·1	—
14	75·0	156	34·1	48·7	17·2	—
21	84·5	196	29·7	48·2	21·0	1·1*

* $E^{1\%}_{1\,cm.}$ at 268 mμ. was 6 and values less than 5 in other cases were neglected.

Table 4. *Actual weights of the individual component fatty acids formed by* Aspergillus nidulans *during different periods of its development in* 175 *ml. of medium*

Time of incubation (days)	Acids (g.)			
	Saturated	Oleic	Linoleic	Linolenic
5	0·15	0·25	0·10	—
7	0·21	0·46	0·13	—
9	2·09	2·80	1·01	—
14	1·74	2·48	0·88	—
21	1·13	1·83	0·80	0·04

DISCUSSION

Hilditch (1951) has suggested that syntheses of both saturated and unsaturated fatty acids take place separately, but possibly both are derived from a common precursor. He points out that the 'desaturation' hypothesis is based merely on the interpretation on a percentage basis of the experimental results of workers who were following changes proceeding in ripening seeds, and he shows that if such results are calculated in terms of the actual weights of the fatty acids synthesized at a given time in the same number of seeds, then the quantities of all the acids are seen to increase with the development of the seeds. As an instance, he quotes figures, obtained by Grindley (1950) while studying the development of cotton seed, to show that a decrease observed in the percentage of oleic acid did not necessarily mean that the actual amount of that acid had decreased.

SUMMARY

1. The rate of formation of fat in surface cultures of *Aspergillus nidulans*, when cultivated under the conditions stated, is slow during the first week of incubation, but becomes very rapid during the eighth and ninth days, when the maximal development of the mould is reached. After the ninth day the reserve of fat in the mould decreases in weight.

2. In the early stage of fat synthesis the fat is more acidic and contains a larger percentage of unsaponifiable substances than at any later period.

3. Until the stage of maximal activity of the mould is attained the actual amounts of both saturated and unsaturated component acids continue to increase, but in the subsequent phase of the life of the culture the weight of each of these components diminishes.

4. The results are not in accord with the hypothesis that, for the biosynthesis of fat, unsaturated acids are provided by dehydrogenation of saturated acids.

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The Composition of the Extracellular Polysaccharides of *Aerobacter–Klebsiella* Strains

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Since the earliest observations on coliform bacteria it has been known that organisms of the *Aerobacter–Klebsiella* group (i.e. *Aerobacter aerogenes*, *Aero. cloacae* and *Klebsiella* species) produce large amounts of slime and capsular material, and thus show mucoid growths. The polysaccharide nature of the slime and capsular material of these organisms was established by Emmerling (1900), Schardinger (1902), Toenniessen (1921) and Kramár (1922), and preliminary analyses of untyped strains showed galactose to be present in each. However, few detailed chemical studies have been made. The capsular polysaccharides of *Klebsiella pneumoniae* types A, B and C (corresponding to *Klebsiella* types 1, 2 and 3 of Edwards & Fife, 1952) have been examined more thoroughly and found to be glucose-containing polyuronides (Heidelberger, Goebel & Avery, 1925; Goebel & Avery, 1927; Goebel, 1927). Wilkinson, Dudman & Aspinall (1955) isolated the slime of *Klebsiella* type 54 (*Aero. aerogenes* A 3 (Sl)) from culture supernatants, and found it to be a polyuronide consisting of D-glucose (50%), L-fucose (10%) and an unidentified uronic acid (29%); a small amount of galactose (approx. 1%) detected in the slime was believed to be present only through contamination with an intracellular galactan. The composition of the slime was shown to be independent of the carbon source on which the organism had been grown. Recently, Levine, Stevenson, Bordner & Edwards (1955) examined the capsular polysaccharides of most known *Klebsiella* types by infrared spectrophotometry and found them all to be polyuronides, in some cases with esterified acetyl groups. No analysis has been reported of polysaccharides of *Aero. cloacae* strains.

Mucoid strains of *Aerobacter–Klebsiella* organisms have been differentiated into a very large number of immunologically distinct types (Edwards & Fife, 1952; Edmunds, 1954; Henriksen, 1954) whose specificities are determined by their capsular polysaccharide antigens (Julianelle, 1926; Edwards, 1929). These results indicate that, as with the pneumococcus, *Aerobacter–Klebsiella* strains of different serological type give rise to immunologically distinct extracellular polysaccharides. Levine *et al.* (1955) found the infrared spectrum of the capsular polysaccharide of each type to be different and distinct.

No attempts appear to have been made in previous studies of bacterial polysaccharides to isolate slime and capsular material separately in

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order to establish their relationship by comparative analysis. The view has been held that slime and capsule are of different origin, that slime is a secretion, whereas capsules arise through cell-wall swelling (Etinger-Tulczynska, 1933), but it is now generally believed that both slime and capsule are secretion products (see Dubos, 1945; Knaysi, 1951; Lamanna & Mallette, 1953). It has been shown with pneumococcus type III (Wood & Smith, 1949) and *Aerobacter–Klebsiella* strains (Edwards & Fife, 1952; Wilkinson, Duguid & Edmunds, 1954) that the slime and capsule of capsulate strains are identical antigenically. However, the chemical identity of slime and capsular polysaccharide has yet to be shown.

In the present investigation the composition of the extracellular polysaccharides of *Klebsiella* strains, representing five immunologically different types (types 8, 26, 29, 54 and 57 of the Edwards & Fife classification) was studied to find whether they differed from type to type. In addition, the slime and capsule of *Klebsiella* type 54 (strain A 3) were separately isolated and analysed. The extracellular polysaccharides of two strains of *Aero. cloacae* were also examined.

ORGANISMS AND METHODS

Organisms. *Klebsiella* strains A 1, A 3, A 4 and A 29 are capsulate strains isolated in this Department by Dr J. P. Duguid. Their biochemical and morphological characters are described by Edmunds (1954), who found them to be identical with known *Klebsiella* types of Edwards & Fife (1952). Strains A 1 and A 3 correspond with type 54, strain A 4 with type 8, and strain A 29 with type 57. Although strains A 1 and A 3 are identical antigenically, they differ in their characteristic capsule size, that of A 1 being much larger (Wilkinson *et al.* 1954). Strains E 26 (Sl) and E 29 (Sl) are non-capsulate slime-forming mutants isolated by Dr P. N. Edmunds from types 26 and 29 of Edwards & Fife. *Aero. cloacae* strains NCTC 5920 and NCTC 5936 were obtained from the National Collection of Type Cultures, Colindale, London; under most conditions they are non-capsulate organisms, producing small amounts of slime. The distribution of polysaccharide production in some of these strains has been studied by Duguid & Wilkinson (1953) and Wilkinson *et al.* (1954).

Cultural conditions. The organisms were grown in a simple synthetic medium of the following percentage composition: glucose, 1·0; $(NH_4)_2SO_4$, 0·03; Na_2HPO_4, 1·0; KH_2PO_4, 0·3; K_2SO_4, 0·1; NaCl, 0·1; $MgSO_4,7H_2O$, 0·02; $CaCl_2,6H_2O$, 0·002; $FeSO_4$, 0·0001 (all w/v). This medium contained the relatively high sugar and low nitrogen-source concentrations found to give maximal polysaccharide production (Duguid & Wilkinson, 1953; Wilkinson *et al.* 1954).

Initially, growth was carried out in Roux bottles in solid medium obtained by adding 2% of agar to the medium, but the use of solid medium was discontinued because of the risk of contaminating the polysaccharides with agar. This became especially important when small amounts of galactose were detected in some of the polysaccharide hydrolysates. Growth in aerated liquid medium, after inoculation with loopfuls of 24 hr. cultures, was carried out in 1 l. screw-capped bottles for 48 hr. at 35° in the manner described by Wilkinson *et al.* (1955).

Isolation and purification of the polysaccharides

Slime. The procedure for isolating slime polysaccharide was the same for capsulate and purely slime-forming strains. The liquid cultures were centrifuged at 13 000 rev./min. for 30–60 min. and the supernatants decanted and collected. Owing to the viscosity of the cultures it was not possible at this stage to remove completely all the cells, especially with strains producing large amounts of slime. The precipitation of the polysaccharide from the supernatant with acetone and the subsequent purification and final isolation of the purified material were carried out in the manner described by Wilkinson *et al.* (1955, method C).

Capsule. The capsular polysaccharide of capsulate strains was isolated from the cell residues collected from the initial centrifuging described above. Before decapsulation the cells were washed twice with equal volumes of water to remove any adherent slime. Unsuccessful attempts to remove the capsules of *Klebsiella* strain A 3 by shaking cells in the cold with water or NaCl solutions of up to 20% (w/v) concentration indicated that more drastic extraction conditions were necessary. Two different procedures were found to remove capsules successfully: (*a*) Boiling: cells were suspended in several vol. of water, the pH was checked and adjusted to 7·0 if necessary, and the suspension then boiled for 15 min. (*b*) Alkaline extraction: cells were suspended in 2–3 vol. of 1% (w/v) formaldehyde solution and set aside for 15 min. NaOH was added to a final concentration of 1% (w/v) and the suspension stirred at room temperature for 30 min. and then neutralized with N-HCl. The formaldehyde pretreatment was found to reduce cell disruption considerably, as shown by determining the amount of Kjeldahl N released by alkaline extraction from both pretreated and unpretreated cells. In the cells pretreated with formaldehyde, 25% of the total Kjeldahl N was in the supernatant fraction, but in the unpretreated cells the N value of the supernatant increased to 52%.

The boiled or NaOH-extracted suspension was then centrifuged and the sediment of decapsulated cells discarded. The capsular polysaccharide was precipitated from the supernatant by addition of acetone (2–3 vol.), the mixture being stirred with a spatula, when it was found that the polysaccharide adhered to the spatula as a stringy mass. This produced a partial purification from the intracellular polysaccharide which formed a flocculent precipitate which did not adhere to the spatula. The precipitate was then purified in the same way as the slime polysaccharides. The capsular polysaccharide of strain A 3 was isolated by both extraction procedures, and the boiled preparation found to be the purer product, as indicated by lower N and P values. All the other capsular polysaccharides were therefore isolated by the boiling method.

General analytical methods

The total N, total P, ash, reducing and anthrone values were determined by the methods described by Wilkinson *et al.* (1955). In addition to the 'anthrone equivalents' given previously, that of mannose was found to be 0·52.

Optical rotation. This was determined on 0·1% polysaccharide solutions in water, 1 dm. tubes being used. The turbidity of the solutions made it necessary to use this low polysaccharide concentration.

Identification and determination of component sugars

Glucose, galactose, fucose and mannose. These sugars were identified and estimated by the standard methods of paper chromatographic techniques applied to polysaccharide analysis. The polysaccharides were all hydrolysed under the same conditions, i.e. 24 hr. in 96% (w/v) formic acid at 100°, the formic acid was distilled off under reduced pressure, and the syrup further hydrolysed for 6 hr. in N-H_2SO_4 at 100°. The details are as described by Wilkinson *et al.* (1955).

For identification of the component sugars, the hydrolysates were neutralized with $BaCO_3$ and centrifuged and the supernatants evaporated to syrups. These were dissolved in small amounts of water and suitable small volumes applied to the starting lines of Whatman no. 1 chromatogram strips. The solvents used were *n*-butanol–ethanol–water mixture (5:1:4, by vol.) and *n*-butanol–acetic acid–water mixture (4:1:5, by vol.); irrigation with these solvents was carried out at room temp. for 96–120 hr. and 24–48 hr. respectively. Each hydrolysate was examined on replicate chromatograms, which were sprayed with a variety of reagents in order to detect different groups of sugars: (*a*) saturated aqueous aniline oxalate; (*b*) 1% (w/v) naphthoresorcinol in 0·25N-HCl; (*c*) the Morgan & Elson reagents (Partridge, 1948). Spots were identified by comparing their rate of movement, and colour, with those of monosaccharide standards run on the same chromatogram strips. In this way glucose, galactose, fucose and mannose were detected and identified.

For determination of the component sugars, the polysaccharides (15–30 mg. samples) were hydrolysed as described above, the uronic acid component was precipitated and removed as the Ba salt and the analysis carried out by the procedure used by Wilkinson *et al.* (1955). The reference sugar (15–30 mg.) used in these determinations depended on the composition of the polysaccharide; when mannose was present ribose was used, and when mannose was absent arabinose was used. These sugars were chosen because they could be easily separated from those in the hydrolysates.

Fucose. This was also determined separately on the unhydrolysed fucose-containing polysaccharides (75–100 μg. samples) by the colorimetric method of Dische & Shettles (1948) with the corrections described by Wilkinson *et al.* (1955).

Uronic acid. This was determined on the unhydrolysed polysaccharides by the decarboxylation method of McCready, Swenson & Maclay (1946). By trapping the CO_2 in 0·02N-NaOH and back-titrating with 0·02N-HCl, it was possible to carry out determinations on 20–30 mg. samples of polysaccharide. Control determinations with glucurone (20 mg.) gave reproducible recoveries of 104%.

Serological methods

Antiserum. Cultures of *Klebsiella* strain A3 were grown for 24 hr. at 35° on agar containing 0·5 g. of lactose and 0·5 g. of peptone/100 ml. Whole-culture suspensions (cells, capsule and slime) were made in 0·85% (w/v) NaCl solution to a density of about 10^9 cells/ml. Rabbits were injected with such suspensions to provide antiserum for *Klebsiella* strain A3. The antiserum, which was prepared and kindly provided by Dr P. N. Edmunds, was the same as that used by Wilkinson *et al.* (1954).

Precipitation tests. These were made to demonstrate the reaction of antiserum with solutions of some of the *Klebsiella* polysaccharides. Doubling dilutions of the antiserum in 0·85% (w/v) NaCl solution, from 1/2 to 1/32, were placed in narrow tubes and a polysaccharide solution (0·1% (w/v) in 0·85% (w/v) NaCl) superimposed. The tubes were incubated for 15 min. at 35° and examined, when a positive reaction was indicated by the appearance of a white ring at the interface.

Precipitin-absorption tests. The absorbing reagent (0·1%, w/v, polysaccharide solution in 0·85%, w/v, NaCl solution) was added dropwise with constant agitation to an equal volume of antiserum, and the mixture incubated at 35° for 30 min. After removal of the precipitate by centrifuging, the supernatant was tested with the appropriate polysaccharide solution to find whether precipitating activity had been lost as a result of absorption. If activity remained, more of the polysaccharide solution was added until absorption was complete.

RESULTS

The following polysaccharides were isolated, purified and examined; the slime of *Klebsiella* type 54 (A3), type 26 and type 29, and *Aero. cloacae* strains NCTC 5920 and NCTC 5936, and the capsular polysaccharide of *Klebsiella* type 54 (A1 and A3), type 8 (A4) and type 57 (A29). The yields of polysaccharide varied with strain and batch. For slime, the yields were of the order of 0·05–0·1 g./l. for *Klebsiella* type 54 (A3) and *Aero. cloacae* strains NCTC 5920 and NCTC 5936, and 0·2–0·3 g./l. for *Klebsiella* types 26 and 29. The yields of capsular polysaccharide varied between 0·2 and 0·4 g./l.

The polysaccharides were isolated as grey–white stringy masses. They were highly hygroscopic, absorbing moisture rapidly to a maximum of 15% in 4 hr., and when moistened swelled into gels. They dissolved in water with difficulty, in concentrations up to 1–2%, to give viscous solutions.

The homogeneity of the polysaccharide preparations was established by examination on the ultracentrifuge, 0·25% (w/v) solutions in 0·2M-NaCl being used. Their sedimentation diagrams indicated single molecular species in each preparation, with narrow molecular-weight distributions (Dudman & Greenwood, unpublished results).

Chemical composition

All the polysaccharides were found to contain uronic acid. With the exception of the slime of *Aero. cloacae* NCTC 5936, they were analysed quantitatively, and the results are given in Table 1.

At least two samples of each polysaccharide were hydrolysed, and the component sugars in each hydrolysate estimated in duplicate or triplicate. The results in Table 1 are expressed to the nearest whole number; the accuracy of the data relating to the component sugars is indicated by the closeness of the theoretical and experimental anthrone values. The components of the slime of *Aero.* Rao, 1951) and ethyl acetate–pyridine–water mixture (5:2:5, by vol.), but the results were unsuccessful. The specific colorimetric tests devised by Dische (1947, 1948) for distinguishing between glucuronic and galacturonic acids were carried out on hydrolysates of *Klebsiella* type 54 polysaccharides, with ambiguous results. The uronic acid components remain unidentified.

Table 1. *Analytical results on the polysaccharides of* Klebsiella (*and* Aerobacter cloacae) *strains*

Klebsiella type	54			8	57	26	29	
Strain	A3		A1	A4	A29	E26 (Sl)	E29 (Sl)	NCTC 5920
Nature of polysaccharide	Slime	Capsule	Capsule	Capsule	Slime	Slime	Slime	Slime
Method of extraction	—	Boiling NaOH	Boiling	Boiling	—	—	—	—
Glucose (%)	47	43 37	48	22	0	35	0	17
Galactose (%)	1	2 4	1	51	21	26	31	26
Fucose (%)								
(*a*) Chromatographically	8	9 6	10	0	0	0	0	25
(*b*) Colorimetrically	9	9 7	11	—	—	—	—	28
Mannose (%)	0	0 0	0	3	44	23	13	0
Uronic acid (%)	30	27 26	29	25	28	17	25	22
Total percentage of sugar components	87	81 74	89	101	93	101	69	93
Reducing value of unhydrolysed polysaccharide (%, as glucose)	1·2	0·9 0·6	0·7	0·7	0·7	0·8	1·0	1·6
Anthrone value (%, as glucose)	59	59 54	60	52	40	55	40	58
Anthrone value theoretical (%, as glucose)	57	54 47	60	55	37	60	44	57
Ash (%)	7·8	6·2 9·2	5·5	2·3	3·2	5·0	8·9	4·3
N (%)	0·08	0·53 0·88	0·46	0·65	0·59	0·12	0·30	0·30
P (%)	0·46	0·11 0·38	0·02	0·25	0·02	0·59	1·08	0·19
$[\alpha]_D^{15}$	−59°	* −82°	−77°	+95°	+220°	+138°	+128°	+110°

* Solution too turbid to read.

Table 2. *Highest dilution of antiserum giving precipitation reactions with various polysaccharides of* Klebsiella *type* 54

Antigen (polysaccharide)	A3 antiserum absorbed with					
	Nil	A1	A3 slime	A3* capsule	A3 (Sl)*	A3 (0) intracellular
A1	16	0	0	0	0	16
A3 slime	16	0	0	0	0	16
A3 capsule†	16	0	0	0	0	16
A3 (Sl)*	16	0	0	2	0	16
A3 (0) intracellular	8	8	8	4	8	0

* Freeze-dried preparation (Wilkinson *et al.* 1955). † Alkali-extracted.

aerogenes NCTC 5936 were identified as glucose, galactose, fucose and a uronic acid (21 %, estimated by the colorimetric method of Jarrige, 1947).

Attempts were made to identify the uronic acids of all the polysaccharides, by running the qualitative hydrolysates on chromatograms, together with standards of glucuronic, galacturonic and mannuronic acids, using solvents reported to be suitable for this purpose, e.g. ethyl acetate–acetic acid–water mixture (3:1:3, by vol.) (Rao, Beri &

Serological results

The immunological relationship of the following polysaccharides of *Klebsiella* type 54 strains were examined: capsule of A1, slime of A3, capsule of A3, slime of the non-capsulate mutant A3 (Sl) (described by Wilkinson *et al.* 1955), and an intracellular polysaccharide isolated from the non-slime-forming, non-capsulate mutant, A3 (0). (The origin and characteristics of this mutant have

been described by Wilkinson *et al.* 1954; the isolation and composition of the polysaccharide will be described in a later communication.) The results of the precipitation and precipitin-absorption tests, given in Table 2, show that the slime and capsule of A 3, the slime of A 3 (Sl) and the capsule of A 1 are antigenically similar or identical, confirming the findings of Wilkinson *et al.* (1954). They also show that the capsular polysaccharide of A 3 contained serologically detectable amounts of the A 3 (0) intracellular polysaccharide.

The high degree of serological activity of these purified polysaccharide preparations was demonstrated by titrating the A 3 antiserum (diluted 1/4) against increasing dilutions of A 3 (Sl) slime polysaccharide in 0·85% (w/v) NaCl, when positive results were obtained in the precipitation ring test with slime dilutions up to $1:10^6$.

DISCUSSION

The extracellular polysaccharides of the six strains of *Klebsiella* and two strains of *Aerobacter cloacae* examined were all found to be polyuronides, and they thus conform to the general pattern of bacterial extracellular heteropolysaccharides, most of which are known to possess acidic or basic component sugars. The results support the findings of Levine *et al.* (1955), based on infrared spectrophotometric evidence, that the capsular polysaccharides of all *Klebsiella* types contain uronic acids. All the polysaccharide samples examined here contained small amounts of nitrogen-containing material, which was shown not to be hexosamine, and was most probably the result of contamination with protein and nucleic acids.

Galactose was present in small amounts in the polysaccharides of *Klebsiella* type 54 strains (A 3 slime, A 3 capsule and A 1 capsule), but was most likely due to contamination with some intracellular polysaccharide. This view is supported by the results of the precipitin-absorption tests, which show that the A 3 capsular preparation containing the highest amount (4%) of contaminating galactose, when used to absorb anti-A 3 antiserum, slightly reduced the titre against the A 3 (0) intracellular polysaccharide. Preliminary analysis of this latter polysaccharide has shown it to be a galactan (Dudman & Wilkinson, unpublished results).

Fucose was unknown in bacteria until recently found in the polysaccharides of *Bacillus polymyxa* (Misaki, Hori & Teramoto, 1954), *Lactobacillus bifidus* (Norris, de Sipin, Zilliken, Harvey & György, 1954), *Klebsiella* type 54 strain A 3 (Sl) (Wilkinson *et al.* 1955), *Salmonella poona*, *Salm. grumpensis* and *Chromobacterium violaceum* (Davies, 1955). In the present study fucose was found in the polysaccharides of other strains of *Klebsiella* type 54 and the two strains of *Aero. cloacae*. Good correlation was obtained in the fucose determinations by the colorimetric and chromatographic methods.

The present results show that the five *Klebsiella* types produce extracellular polysaccharides which differ markedly in composition from each other. It will be seen from Table 1 that the polysaccharide of each type is qualitatively or quantitatively different from those of the other types studied here. Thus, in these *Klebsiella* types, immunological differences are accompanied by considerable differences in composition of the polysaccharide antigens. In many cases of immunological cross-reactions between unrelated organisms it has been found that the polysaccharide antigens contain uronic acid components. It is therefore believed that uronic acids dominate the specificity of antigens in which they are present, and that, when their steric arrangement in different antigens is sufficiently similar, cross-reactions take place. The absence of cross-reactions between any of the *Klebsiella* types studied here (see Edwards & Fife, 1952), despite the similar amounts of uronic acid present in the polysaccharide antigen of each type, indicates that the steric arrangement of uronic acid in each polysaccharide must be different, most probably as a result of the different composition of each polysaccharide.

By separately isolating and analysing the slime and capsular polysaccharide of *Klebsiella* type 54 strain A 3, it was shown that they are identical in composition. This is readily seen from the relative proportions of the component sugars, presented in Table 3. From the same table it can be seen that the polysaccharides of strains A 3 (Sl) and A 1 are identical in composition with A 3 polysaccharides The differences in $[\alpha]_D$ values (see Table 1) are not regarded as significant because the actual polarimetric readings were sufficiently small for differences of this magnitude to arise as a result of experimental error. Thus it has been shown that

Table 3. *Molecular ratios of component sugars in the extracellular polysaccharides of* Klebsiella *type* 54 *strains, relative to glucose as* 100

	Component sugars		
Polysaccharide	Glucose	Uronic acid*	Fucose†
A 1 capsule	100	56	25
A 3 slime	100	59	21
A 3 capsule‡	100	58	23
A 3 (Sl) slime§	100	54	22

* Assumed to be hexuronic acid.
† Calculated from colorimetric values.
‡ Extracted by boiling.
§ Freeze-dried preparation (Wilkinson *et al.* 1955).

the slime and capsule of a capsulate strain, and the slime of the non-capsulate slime-forming mutant derived from it, are chemically identical. Further proof of chemical identity, however, must await structural investigations and molecular-weight determinations.

The compositional and immunological identity of the polysaccharides of A 1, A 3 and A 3 (Sl) is of particular interest, in view of the results of Wilkinson *et al.* (1954), who found large physical differences between the capsules of A 1 and A 3 and the slime of A 3 and A 3 (Sl). Similarly, Macpherson, Wilkinson & Swain (1953) found differences in the ability of these polysaccharides, after prior NaOH treatment, to inhibit haemagglutination by the influenza group of viruses. Thus the capsular and slime polysaccharides of strain A 3 behaved similarly in inhibiting haemagglutination of PR 8 influenza virus at a concentration of 2·5 μg./ml., and a concentration of 333 μg./ml. of A 1 polysaccharide was required. A 3 (Sl) slime polysaccharide was completely non-inhibitory. Although all these type 54 polysaccharides were absorbed on the red-cell surface, it was found that there was a relationship between the degree of inhibition and the lowering of the red-cell surface charge, presumably owing to ionization of the carboxyl groups of the uronic acids of the polysaccharides. Thus there again appears to be some physical difference in the various polysaccharides, according to the availability of carboxyl groups for ionization. The production of the same polysaccharide in different physical states by closely related strains is one of the many problems about the apparently simple process of capsule and slime formation for which there is at present no explanation. Despite the advances made in the study of the chemical composition and factors influencing the production of bacterial polysaccharides, little is known as to why extracellular polysaccharides are produced in some strains as slime and capsule and in others as slime alone. Any hypothesis must account for the facts that in a capsulate strain the slime and capsule are composed of the same polysaccharide, and that by an apparently single mutable step a capsulate strain may give rise to a non-capsulate slime-forming mutant producing the same polysaccharide. It is possible that the problem of capsule formation may be finally elucidated when observations are made to discover what happens when a slime-forming capsulate strain becomes a slime-forming non-capsulate mutant.

SUMMARY

1. The extracellular polysaccharides of six *Klebsiella* strains, corresponding to five immunological types (types 8, 26, 29, 54 and 57), and two strains of *Aerobacter cloacae* (NCTC 5920, NCTC 5936), were isolated and purified, and shown to be complex polyuronides.

2. The polysaccharide of each *Klebsiella* type was found to be qualitatively or quantitatively different in composition from those of other types.

3. The slime and capsular polysaccharides of *Klebsiella* type 54 strain A 3 were separately isolated and analysed, and found to be identical in composition and immunological specificity.

4. The chemical and immunological relationship of the extracellular polysaccharides of *Klebsiella* type 54, strains A 1, A 3 and A 3 (Sl), was examined and they were found to be identical.

5. The slime polysaccharide of strain A 3 (Sl) in dilutions of $1:10^6$ reacted with homologous immune serum.

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The Fate of ^{131}I-Labelled Homologous and Heterologous Thyroglobulins in the Rat, Dog, Monkey and Rabbit

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Iodine is readily fixed by the thyroid gland, where it is rapidly incorporated into thyroglobulin. If radioactive iodide is administered to an animal the gland may be damaged and total destruction can easily be accomplished. Thyroglobulin from the damaged tissue passes into the circulation, where its presence has been recognized by means of chromatographic and ultracentrifugal methods (Tong, Taurog & Chaikoff, 1952; Robbins & Rall, 1952; Brown & Jackson, 1954; Robbins, Peterman & Hall, 1954). Examination of the plasma of patients with thyroid carcinoma after treatment with large doses of ^{131}I (approx. 150–200 mc) has shown that a range of patterns of radioactive components occurs (Brown & Jackson, 1954). These appear to be related to a varying ability of different individuals to metabolize the thyroid protein liberated into the blood as a result of the destructive effects of the radioactive iodide. It seemed of interest therefore to study the metabolism of thyroglobulin in experimental animals. In the present paper, results are described from an examination of the fate of ^{131}I-labelled homologous and heterologous thyroglobulin in various species. A preliminary account of some of this work has already been published (Brown & Jackson, 1955).

EXPERIMENTAL

Preparation and administration of labelled thyroglobulin. At a suitable interval after the injection of carrier-free [^{131}I]iodide, animals were killed, the thyroid glands removed, ground with powdered glass with additions of ice-cold water and centrifuged. The precipitate, which invariably contained less than 5% of the total radioactivity of the gland, was discarded. The supernatant was stored at −20°. Over 99% of the radioactive iodine in these preparations was protein-bound, and paper-electrophoretic examination indicated the presence of a single radioactive component which, in the presence of plasma, was associated with the α-globulins.

Portions of the appropriate thyroglobulin solution containing 50–5000 μg. of protein/kg. body wt. were injected intravenously into animals and samples of venous blood obtained at suitable intervals. In rats, the tissue distribution of radioactivity after the administration of thyroglobulin was studied in groups of animals which were killed at various times. The tissues under investigation were rapidly removed and digested in LiOH solution (10 g. of LiOH, 1 g. of KI in 100 ml. of 20%, v/v, ethanol) and portions counted in a M6 liquid counter. Plasma and whole-blood samples (0·1 ml.) were dried on aluminium trays and their radioactivity was measured directly under a mica end-window counter.

Chromatography and fractionation procedures. Portions (10 μl.) of plasma, to which marker diiodotyrosine, triiodothyronine and thyroxine had been added, were analysed by ascending chromatography on Whatman no. 1 paper in butanol–dioxan–2N NH_3 (4:1:5, by vol.). The position of the marker substances was ascertained by developing with ninhydrin; the paper was then cut into small sections and the radioactivity measured under an end-window counter. In some instances, plasma samples were fractionated by a procedure which has already been described (Brown & Jackson, 1954). This involved the treatment of plasma with silver phosphate (hence estimation of iodide) and precipitation of the proteins with methanol (hence determination of the radioactivity present as thyroglobulin).

RESULTS

All the results described refer to ^{131}I-labelled thyroglobulin.

Metabolism of rat thyroglobulin

The clearance of this protein from the plasma of the rat, dog, monkey and rabbit that follows the intravenous administration of rat thyroglobulin is

shown in Fig. 1. In the rat and monkey the plasma thyroglobulin fell rapidly, whilst thyroxine and iodide appeared (Table 1). Injection of rat thyroglobulin into rats in which the thyroid gland had been ablated by treatment with ^{131}I some weeks before gave similar results. In the dog and rabbit, after an initial sharp drop, rat thyroglobulin disappeared more slowly, and only traces of radioactive metabolites were detected (Table 1). The removal of thyroglobulin from the circulation of all the species was independent of the amount of protein administered over a wide range; for example, essentially similar curves were obtained in the rat when amounts of radioactive protein ranging from that present in an entire rat gland down to 1% of this amount were injected. A second injection of rat thyroglobulin into the rabbit or dog led to its very rapid elimination from the plasma, presumably owing to the development of immunity. Further evidence of this is summarized in Table 2, where the change in distribution of radioactivity between plasma and cells in normal and immune animals is shown (cf. Almond, Francis, Hawkins & Wormall, 1954). Repeated injections of rat thyroglobulin into rats or monkeys, however, failed to increase the clearance rate of the protein from the blood. A preliminary study of the tissue distribution of radioactivity in rats after the injection of homologous thyroglobulin gave no indication of preferential localization.

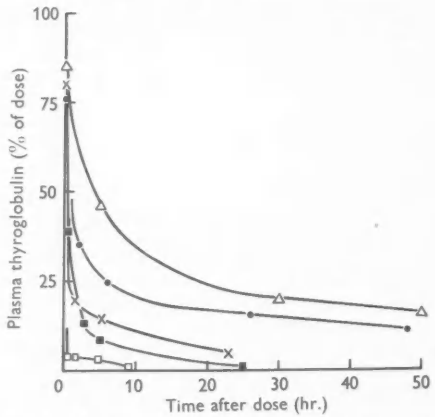

Fig. 1. Clearance of ^{131}I-labelled rat thyroglobulin from the plasma of the rat (×), dog (●), monkey (■) and rabbit (△) after one intravenous injection of the protein. The disappearance of rat thyroglobulin from the plasma of a dog previously immunized to this protein is also shown (□).

Metabolism of dog thyroglobulin

The disappearance of dog thyroglobulin from the plasma after intravenous injection into various species is shown in Fig. 2. Elimination of radioactivity was rapid in the dog, but was considerably slower in other species. The clearance of the protein in the dog was accompanied by the appearance of thyroxine, iodide and traces of iodotyrosines. The presence of iodinated tyrosine derivatives in the plasma is a further indication of the extra-thyroidal hydrolysis of homologous thyroglobulin since these amino-acids are not normally found in plasma. Apart from traces of thyroxine in the rat, no radioactive metabolites were detected after the in-

Table 1. *Distribution of radioactive components in the plasma of various species after the intravenous injection of rat thyroglobulin labelled with* ^{131}I

Recipient species	Time after injection (hr.)	Radioactive dose in plasma (%)	Distribution of radioactivity in plasma (%)			
			Iodide	Iodotyrosines	Thyroxine	Protein
Rat	1·5	20·4	3	1	1	95
	5·5	18·6	17	0	6	77
	23	5·8	6	0	18	76
	95	1·3	20	0	26	54
Monkey	0·16	39·2	0	0	1	99
	2·8	16·1	0	0	18	82
	5	11·1	5	0	19	76
	24	4·3	—	—	—	—
Dog	2	38·4	2	5	2	91
	6	27·2	4	3	3	90
	26	16·8	7	1	2	90
	48	12·0	5	2	2	91
	100	5·7	2	2	1	95
Rabbit	5	45·7	0	0	1	99
	30	19·3	0	0	1	99
	50	15·6	0	0	2	98
	74	10·5	0	0	2	98
	98	7·3	0	0	3	97
	122	3·9	—	—	—	—

Table 2. *Examples of the distribution of radioactivity between the plasma and cells in various species after the injection of* ^{131}I*-labelled thyroglobulins*

Thyroglobulin injected	Recipient species	Time after injection (hr.)	Radioactivity in plasma / Radioactivity in cells
Rat	Normal rabbit	4·5	3·3
		25	3·3
	Immune rabbit	3·5	0·82
		25	1·2
Rabbit	Normal monkey	1·5	5·9
		6·5	3·3
	Immune monkey	4·0	1·5
Dog	Normal rat	0·3	4·1
		2·0	4·4
		4·0	4·3
	Immune rat	0·5	1·5
		2·0	1·4
		4·0	1·7

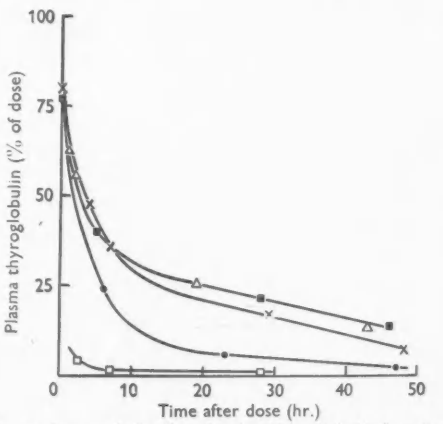

Fig. 2. Removal of radioactive dog thyroglobulin from the plasma of the dog (●), rat (×), monkey (■) and rabbit (△) after a single intravenous injection of the protein. A curve showing the response in the rats previously immunized to dog thyroglobulin is also shown (□).

jection of dog thyroglobulin into the other three species examined. This is emphasized by the constant percentage of protein present (Table 3).

Repeated injection of the protein into rats resulted in the development of immunity (Table 2 and Fig. 2). A comparison of the distribution of radioactivity in the tissues of normal and immune rats after the injection of labelled dog thyroglobulin revealed marked differences between the two groups (Table 4). The liver and spleen of the immune group contained considerably more radioactivity than the corresponding tissues of the normal animals. Similar observations were made in the rabbit by Almond *et al.* (1954) after the administration of foreign protein labelled *in vitro* with ^{131}I.

Metabolism of monkey thyroglobulin

Monkey thyroglobulin was rapidly metabolized in both monkey and rat, with the liberation into the plasma of iodide and thyroxine; it was more slowly removed from the plasma of the dog and rabbit (Fig. 3), with little evidence of breakdown products (Table 5). These results, taken in conjunction with those obtained with rat thyroglobulin, may indicate a structural resemblance between these two proteins.

Metabolism of rabbit thyroglobulin

In striking contrast to the fate of homologous thyroglobulins in the other species referred to above, rabbit thyroglobulin disappeared slowly from the blood of rabbits and radioactive metabolites were not detected (Fig. 4). To eliminate the possibility that the rabbit might show an unusual degree of specificity, even towards homologous thyroglobulin, the fate of thyroglobulin liberated from the thyroid gland by a destructive dose of ^{131}I was examined (Fig. 5). No radioactive metabolites were detected in the plasma, and the clearance of this thyroglobulin followed an exponential curve similar to that found in untreated rabbits; the mean half-life of the protein was about 55 hr. The injection of three successive doses (at weekly intervals) of rabbit thyroglobulin into rabbits failed to modify the shape of the curve, but this protein behaved like other heterologous thyroglobulins in the rat and monkey. A similar rate of clearance of rabbit thyroglobulin in the rat was

Table 3. *Distribution of radioactive components in the plasma of various species after intravenous injection of dog thyroglobulin*

Recipient species	Time after injection (hr.)	Radioactive dose in plasma (%)	Distribution of radioactivity in plasma (%)			
			Iodide	Iodotyrosines	Thyroxine	Protein
Dog	0·25	76·2	0	0	0	100
	6	30·0	10	7	3	80
	23	10·3	35	3	6	56
	47	5·6	57	0	7	36
	96	1·0	45	0	30	25
	125	1·2	60	0	22	18
	148	0·9	49	0	36	15
Rat	4	47·6	0	0	0	100
	7	36·4	0	0	2	98
	29	17·4	0	0	4	96
	48	7·4	1	0	3	96
Monkey	5	40·1	0	0	0	100
	28	21·6	0	0	0	100
	46	13·3	2	0	0	98
	123	4·1	—	—	—	—
Rabbit	1	62·5	0	0	0	100
	2	55·5	1	0	1	98
	19	25·5	1	0	1	98
	43	13·2	1	0	1	98
	93	4·5	0	0	0	100

Table 4. *Distribution of radioactivity in the tissues of normal and immune rats after the intravenous injection of radioactive dog thyroglobulin*

Time after injection (hr.)		Dose (%) in 1 g. of tissue / Dose (%) in 1 g. of blood			
		Liver	Spleen	Kidney	Lung
0·5	Normal	0·39	0·12	0·33	0·39
	Immune	4·62	2·45	1·02	0·83
2·0	Normal	0·26	0·15	0·26	0·33
	Immune	0·88	1·19	0·89	0·59
4·0	Normal	0·24	0·13	0·38	0·41
	Immune	0·93	0·59	1·07	0·45

Table 5. *Distribution of radioactive components in the plasma of various species after intravenous injection of monkey thyroglobulin*

Recipient species	Time after injection (hr.)	Radioactive dose in plasma (%)	Distribution of radioactivity in plasma (%)			
			Iodide	Iodotyrosines	Thyroxine	Protein
Monkey	2·5	37·0	5	2	3	90
	5	24·7	4	1	11	84
	24	5·4	20	0	23	57
Rat	6	15·1	6	17	4	73
	24	5·9	15	22	6	57
	50	1·6	—	—	—	—
Rabbit	2·5	35·0	7	0	0	93
	7	21·8	4	0	3	93
	25	12·7	5	0	5	90
	48	7·3	0	0	3	97
Dog	3·5	17·7	2	0	0	98
	23	11·6	2	0	0	98
	47	7·5	4	0	3	93

reported by Scott, Bostick, Shimkin & Hamilton (1949). An immune response was obtained in the monkey after three successive injections of rabbit thyroglobulin; the relation between the radioactivity in the plasma and cells after the first and fourth injection of the rabbit protein into the same monkey is shown in Table 2. However, four injections of rabbit thyroglobulin into rats did not induce immunity.

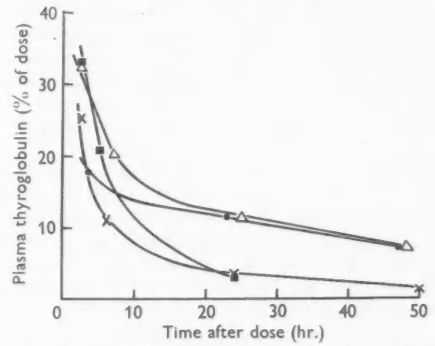

Fig. 3. Clearance of ^{131}I-labelled monkey thyroglobulin (injected intravenously) from the plasma of the monkey (■), rat (×), dog (●) and rabbit (△).

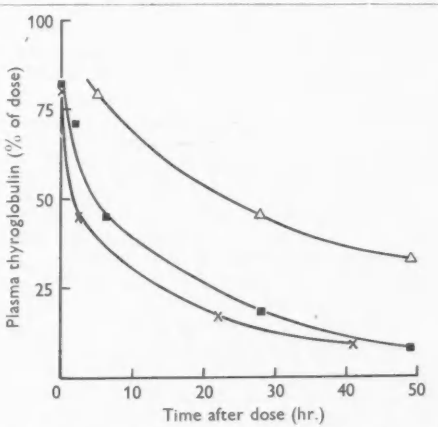

Fig. 4. Removal of intravenously administered ^{131}I-labelled rabbit thyroglobulin from the plasma of the monkey (■), rat (×) and rabbit (△). In the rabbit there is no initial fall in plasma radioactivity such as occurred in the other two species (see also Figs. 1–3).

DISCUSSION

Previous work has established that the destruction of thyroid tissue by the administration of ^{131}I to rats is soon followed by the appearance in the circulation of labelled thyroglobulin, which is then speedily removed (Brown & Jackson, 1954). It has now been shown that the homologous thyroglobulin is removed at a similar rate by both normal and thyroidectomized rats, so that this process is independent of the thyroid gland. Metabolites in the form of thyroxine and iodide (the latter probably arising from deiodination of iodotyrosines) appear in the plasma. Clearly an effective process exists for the specific removal and hydrolysis of homologous thyroglobulin. Administration of the protein over a wide dose range has shown that its

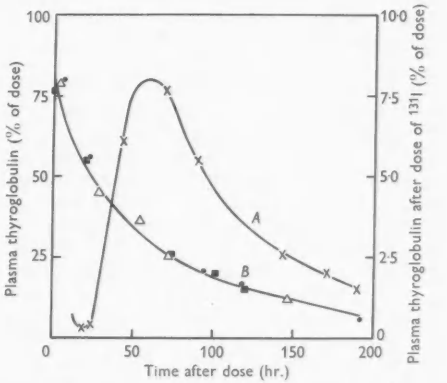

Fig. 5. Radioactive-protein content of the plasma of a rabbit (curve *A*) after a dose of ^{131}I sufficient to destroy its thyroid gland (20 mc). Beyond the peak the curve is identical with that obtained from two other rabbits (△, ●; curve *B*) given an intravenous injection of ^{131}I-labelled rabbit thyroglobulin. The filled squares (■) on curve *B* represent the corresponding points on curve *A*, transposed by modifying the abscissa.

rate of elimination from plasma is independent of the amount injected, and preliminary experiments reveal that liver and splenic tissue can hydrolyse thyroglobulin *in vitro* with the formation of thyroxine and iodotyrosines. It seemed not unreasonable, therefore, to reinvestigate the possibility that thyroglobulin itself might be secreted normally by the gland and then hydrolysed to form thyroxine in the extra-thyroidal tissues. The nature of the circulating thyroid hormone has been a subject for speculation for many years. Hektoen, Carlson & Schulhof (1923), using immunological methods, obtained evidence for the presence of thyroglobulin in the thyroid lymphatics and veins of the goitrous dog; but Lerman (1940), applying similar techniques to man, failed to obtain unequivocal evidence for the presence of thyroglobulin in the thyroid veins under normal

conditions, although trauma to the gland during operation was followed by leakage of the thyroid protein into the circulation. The current view is that thyroxine is secreted as such by the thyroid gland and that thyroglobulin or derived peptides have no physiological role outside the gland (Roche & Michel, 1954).

The possibility that homologous thyroglobulin might normally be metabolized in the extrathyroidal tissues seemed to be strengthened by our observation that rats were unable to metabolize the thyroglobulins of the dog and rabbit, although they rapidly hydrolysed rat thyroglobulins. In fact, rats gave a typically immune response to dog thyroglobulin after two successive injections of this protein (Fig. 2). Similarly, in the dog, homologous thyroglobulin was rapidly degraded into iodide, iodotyrosines and thyroxine, but no such breakdown occurred after administration of the thyroid protein from the rat, rabbit or monkey.

However, there are two objections to the hypothesis that homologous thyroglobulin is normally secreted by the gland. In the first place, the behaviour of monkey thyroglobulin in the rat and of rat thyroglobulin in the monkey was indistinguishable from that of the homologous proteins. A more serious objection is the inability of the rabbit to degrade its own thyroglobulin in the same way that homologous thyroid protein is metabolized in the rat, dog and monkey, especially as the administration of a tracer dose of ^{131}I to the rabbit is followed by the appearance of labelled thyroxine in the plasma. The persistence of homologous thyroglobulin in rabbit plasma without the appearance of metabolites is very similar to the behaviour of heterologous thyroglobulin on first injection into other species, with the exception of the rat–monkey relationship referred to above.

The possibility that a high degree of individual specificity exists towards homologous thyroglobulin in rabbits seems very unlikely, since thyroid protein released into the blood after a destructive dose of ^{131}I disappeared in precisely the same manner as the injected material in other rabbits (Fig. 5). It is interesting that this result parallels that obtained from a patient with carcinoma thyroid after a therapeutic dose of ^{131}I (Brown & Jackson, 1954, case 3). This patient differed from many others in being unable to hydrolyse his own thyroglobulin, which disappeared slowly from the plasma without the formation of thyroxine.

After the first injection of homologous or heterologous thyroglobulins into the rat, dog and monkey (Figs. 1–4) a precipitous fall in the plasma radioactivity occurs during the first 1–2 hr. Three factors are probably involved in this phase: the fixation of radioactive material by erythrocytes (about 20%); the removal of thyroglobulin by metabolism; and, finally, its diffusion into the extravascular space in association with the normal plasma proteins. Paper electrophoresis has shown that thyroglobulin moves with the α-globulin fraction of plasma, and the penetration of the plasma proteins into the extravascular spaces has been demonstrated in the rabbit (Humphrey & McFarlane, 1954). Curiously enough, rabbit thyroglobulin behaves quite differently from that of the rat, dog and monkey. There is neither an initial rapid fall in the blood level nor evidence of hydrolytic breakdown in the rabbit, so that after 5 hr. most of the radioactive protein is still present in the blood (Fig. 4). This behaviour suggests that, in spite of its intimate association with the plasma proteins, rabbit thyroglobulin does not accompany these into the extravascular space. The failure of rabbit thyroglobulin to be metabolized in the rabbit might be accounted for in this way. It is established, however, that rabbit thyroglobulin leaves the plasma steadily with a half-life of about 55 hr., which is considerably shorter than that found for ^{14}C-labelled globulin in the rabbit (Humphrey & McFarlane, 1954).

Repeated injections of homologous thyroglobulin into rabbits failed to modify its rate of removal from the plasma, i.e. no immune type of response was induced. This was not so when heterologous thyroglobulin was used in these animals, for an immune reaction was readily elicited; similar responses to heterologous thyroglobulins occurred in the other species examined, apart from the rat–monkey relationship to which reference was made above. An alteration in the distribution of radioactivity between the plasma and cells always accompanied the immune response to a foreign thyroglobulin (Table 2); this change was found by Almond *et al.* (1954) to be associated with antigen–antibody reactions in the blood. Differences in tissue distribution of radioactive material when dog thyroglobulin is administered to normal and immune rats (Table 4) also provide additional evidence that the response is a true antigen–antibody reaction (compare Almond *et al.* 1954). This is in agreement with the conclusions of Hektoen *et al.* (1923), Stokinger & Heidelberger (1937) and Lerman (1940) that some thyroglobulins can act as antigens.

Gaddum (1929–30), Schulhof (1930) and Clutton, Harington & Yuill (1938) showed that injected ox, human or dog thyroglobulin was able to increase the metabolic rate of rats. Our results, however, suggest that if hydrolysis of the protein into thyroxine is a prerequisite for its action, thyroglobulin from the rat or monkey, but not that from the dog or rabbit, should be capable of increasing the metabolic rate of rats. Experiments designed to test the relative efficiencies of homologous and

heterologous thyroglobulins and thyroxine in increasing the oxygen consumption are in progress.

SUMMARY

1. The metabolism of ^{131}I-labelled thyroglobulin from four mammalian species has been studied.

2. The rat, dog and monkey rapidly metabolize the homologous protein, with the formation of iodide, iodotyrosines and thyroxine. Rabbit thyroglobulin disappears slowly from the plasma of the rabbit after intravenous injection, and no metabolites have so far been detected in the blood.

3. The development of immune reactions to foreign thyroglobulins has been observed in all the species.

4. Heterologous thyroid protein is, in general, cleared from the plasma of non-immune animals of all four species without the appearance in the plasma of significant amounts of simple metabolites. This is not so, however, when monkey thyroglobulin is injected into rats and vice versa, for rapid metabolism takes place and thyroxine appears in the plasma.

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Glutamic Acid Decarboxylase in *Rhodotorula glutinis*

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Enzymic decarboxylation of L-glutamic acid to γ-aminobutyric acid in plants, animals and bacteria is well known, and the importance of this process in nitrogen metabolism has been reviewed (Schales, 1951). Recent observations (Roberts, Ayengar & Posner, 1953) on the transamination of γ-aminobutyric acid with α-oxoglutarate serve to indicate the possible role of glutamic acid decarboxylation in the 'flow of nitrogen'. γ-Aminobutyric acid was first detected in yeast by Reed (1950), who found it in refrigerated samples and autolysates of yeast extract (Difco). The occurrence of γ-aminobutyric acid in a yeast, *Rhodotorula glutinis*, and the glutamic acid decarboxylase activity of washed cell-suspensions of this organism were reported earlier (Krishnaswamy & Giri, 1953). In this paper, details of investigations carried out on this enzyme are briefly described.

METHODS

The organism. The yeast, *Rhodotorula glutinis* Harrison var. *rubescens* Saito (Lodder) was maintained on wort-agar slants by fortnightly subculture.

Cultivation of the organism. To obtain sufficient cell material the organism was cultivated in a synthetic liquid medium (Krishnaswamy & Giri, 1953). A portion (2 l.) of this medium, after sterilization, was seeded with an actively growing inoculum (obtained by making two serial transfers of the organism at 24 hr. intervals) and mildly aerated. The cells were harvested by centrifuging (after growth for 18 hr. at room temp.).

Preparation of the enzyme. In earlier experiments, washed cells suspended in suitable buffers were used directly. Cell-free extracts obtained by grinding the cells with Pyrex-glass powder and centrifuging off the cell-debris showed lower activity. Acetone powders were prepared by treating the cells with a large volume of cold acetone and filtering on a Büchner funnel, after vigorous stirring. After the material had dried, it was finely powdered and stored in a refrigerator. Fresh preparations were always used for the extraction of the enzyme which was done by suspending the acetone powder with 0·02 M Na phosphate buffer, pH 8·0 (20 mg./ml.) for 2 hr. at 30°. The supernatant obtained after centrifuging (3500 r.p.m.) was slightly yellowish in colour and it was directly used.

Measurement of enzyme activity. The reaction mixtures, unless otherwise stated, consisted of 0·2 ml. of 0·1 M L-glutamic acid solution, 0·8 ml. of M/15 acetate buffer,

pH 4·5, and 1·0 ml. of enzyme preparation, total volume being 2·0 ml. They were incubated at 30° and at specified intervals the reaction was arrested by adding ethanol (1 vol.). γ-Aminobutyric acid was estimated in 20-μl. samples of the supernatant by circular-paper chromatography, and interaction with ninhydrin, followed by colorimetric comparison of the colour intensity with that of a standard solution of γ-aminobutyric acid treated in the same way (Giri, Radhakrishnan & Vaidyanathan, 1952). Appropriate blanks and controls which included sets for endogenous reaction were always run. Activity was expressed as μg. of γ-aminobutyric acid formed under specified conditions.

RESULTS AND DISCUSSION

Isolation and estimation of γ-aminobutyric acid by paper chromatography

To 2 ml. of the cell-free extracts various quantities of γ-aminobutyric acid were added from a 0·2% solution, and the total volume was made up to 2·5 ml. Immediately an equal volume of ethanol was added to all the tubes and γ-aminobutyric acid in the supernatants was estimated by paper chromatography. The recovery of added γ-aminobutyric acid from extracts incubated for 1 hr. at 30° was also measured, and the percentage recovery ranged between 94 and 104.

Table 1. *Recovery of added γ-aminobutyric acid from enzyme extracts*

Expt. no.	γ-Aminobutyric acid in the preparation (μg.)	γ-Aminobutyric acid added (μg.)	γ-Aminobutyric acid found in mixture (μg.)	Recovery (%)
1	45	250	285	96
2	45	500	536	98
3	46	1000	1077	103

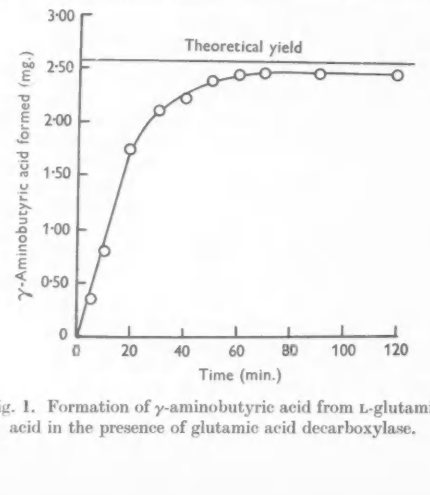

Fig. 1. Formation of γ-aminobutyric acid from L-glutamic acid in the presence of glutamic acid decarboxylase.

After decarboxylation of glutamic acid by the enzyme, γ-aminobutyric acid was isolated by circular-paper chromatography on a preparative scale (Giri, 1954, 1955) from the reaction mixture and the product obtained was compared with a synthetic sample (m.p. and mixed m.p. 192°, nitrogen, 13·1%).

Properties of the enzyme

Experiments showed that the yeast decarboxylase had similar properties to those of bacterial decarboxylase. Glutamic acid decarboxylation was found to be quantitative at pH 4·5 and 30° (Figs. 1–3);

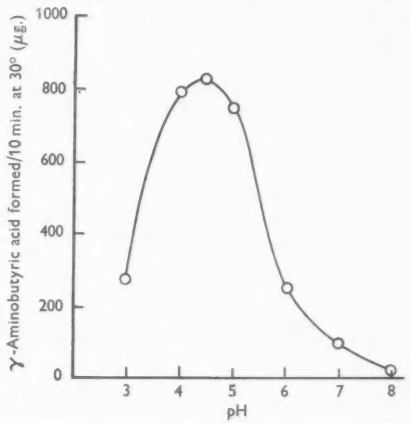

Fig. 2. Effect of pH on the activity of L-glutamic acid decarboxylase.

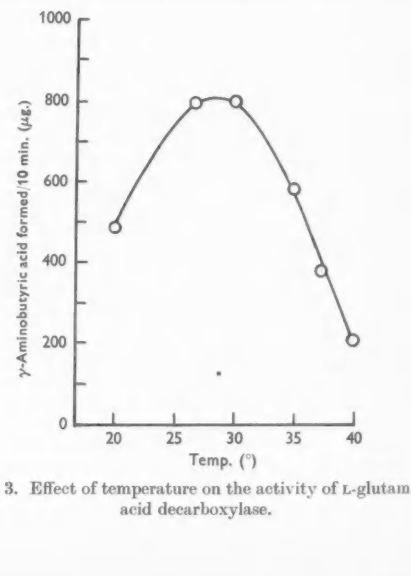

Fig. 3. Effect of temperature on the activity of L-glutamic acid decarboxylase.

the enzyme was unstable above 30°. The Michaelis constant was found to be $2{\cdot}75 \times 10^{-3}$ M. As with other decarboxylases, dialysis reduced the activity and only pyridoxal phosphate was effective in restoring the activity.

Effect of inhibitors. The enzyme was found to be sensitive to silver, cyanide and mercury ions. Hydroxylamine was the most prominent of the carbonyl-trapping reagents. The enzyme did not show any abnormal sensitiveness to copper (the common sulphydryl inhibitor), iodoacetate, or fluoride. In all these respects, the enzyme resembled the bacterial decarboxylase (Gale, 1946).

Reversibility of the inhibition caused by hydroxylamine with pyridoxal phosphate. In one set of reaction mixtures, hydroxylamine was added immediately after the substrate, and in another the enzyme was pre-incubated with the inhibitor for 5 min. at 30°, followed by the addition of the substrate. Pyridoxal phosphate was added to the control (without inhibitor) as well as to other reaction mixtures at the fifteenth minute. It was observed that the addition of pyridoxal phosphate resulted in the reversal of inhibition, γ-aminobutyric acid formation in all the reaction mixtures reaching the optimum level. These observations may serve as additional evidence for an earlier suggestion (Roberts, 1952) that the aldehyde group of pyridoxal phosphate may be the most likely point of attack of the inhibitor as well as of the substrate, the attachment of the latter taking place through the α-amino group, forming a Schiff's base.

Attempts to purify the enzyme

Glutamic acid decarboxylase has not yet been obtained in a purified state. Either acetone powders or crude extracts have been used directly by previous workers (Taylor & Gale, 1945; Roberts & Frankel, 1950, 1951). Attempts to purify the enzyme by fractional precipitation with organic solvents such as acetone and ethanol failed to give satisfactory recoveries of the enzyme. Freshly prepared acetone powder (300 mg.) was suspended in 15 ml. of 0·02 M phosphate buffer, pH 8·0 (preparation *A*). The suspension was kept for 2 hr. at 30° with frequent stirring and the supernatant (12 ml.) obtained after centrifuging off the sediment was preserved (preparation *B*). Acetic acid (3%) was added slowly to the supernatant until the pH reached 5·5. A portion (6 ml.) of a suspension containing 10 mg. of alumina C_γ/ml. was added and the mixture was allowed to stand for 15 min. The adsorbent was centrifuged down and twice eluted with 6 ml. of M/15 phosphate buffer (pH 7·0) and the eluates were combined (preparation *C*). The combined eluates were precipitated with $(NH_4)_2SO_4$ (50 g./100 ml.), the precipitate was dissolved in 10 ml. of water and the salt concentration increased to 55% saturation. The precipitate thus obtained was dissolved in 10 ml. of water, the solution dialysed for 24 hr. in the cold and the volume made up to 15 ml. (preparation *D*). The activity of these preparations was determined as before, 40 μg. of pyridoxal phosphate being added in every case. The results are shown in Table 2.

Table 2. *Progress of purification of* L-*glutamic acid decarboxylase*

Activity is expressed as μg. of γ-aminobutyric acid formed/10 min. at 30°/ml. of enzyme preparation.

Stage of purification (see text)	Activity	Dry wt. (mg./ml.)	Activity/mg.	Degree of purification
A	1192	20·4	58·42	—
B	998	9·2	108·47	1·9
C	650	4·3	151·16	2·6
D	296	1·4	211·42	3·6

SUMMARY

1. Glutamic acid decarboxylase has been detected in a yeast, *Rhodotorula glutinis*, and its properties were studied by the application of circular-paper chromatography.

2. Decarboxylation was quantitative at pH 4·5 and 30°. The Michaelis constant was $2{\cdot}75 \times 10^{-3}$ M. Pyridoxal phosphate could restore the activity of dialysed preparations and reverse the inhibition caused by hydroxylamine.

3. Adsorption of the enzyme (precipitated by acidification of crude extracts) on alumina C_γ and fractional precipitation of the eluted enzyme with $(NH_4)_2SO_4$ have been carried out in an attempt to purify the enzyme.

We are grateful to Dr Deutsch of the California Foundation for Biochemical Research, for his generous gift of the diammonium salt of pyridoxal phosphate.

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The Role of Glutamine in the Biosynthesis of Hyaluronate by Streptococcal Suspensions

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Hexosamine-containing high-molecular-weight substances form major constituents of many animal and bacterial products, yet only during the last few years has considerable attention been paid to the mechanism of their biosynthesis. Hyaluronate has been known for many years to be formed by strains of Lancefield Groups A and C *Streptococcus haemolyticus* (Kendall, Heidelberger & Dawson, 1937; Seastone, 1939). This bacterial mucopolysaccharide is likely to be identical with the mammalian product originally isolated by Meyer & Palmer (1936). It seems, therefore, reasonable to use this convenient system to obtain preliminary information about mucopolysaccharide synthesis. Recently, Roseman, Moses, Ludowieg & Dorfman (1953, 1954) and Topper & Lipton (1953) have studied the structural relationship between the hexosamine and hexuronic acid moieties of hyaluronate, synthesized by cultures of Group A haemolytic streptococci, and the glucose present in the culture medium. They concluded that both hexose derivatives arose without rearrangement of the glucose carbon atoms. Meanwhile, our interest (Lowther & Rogers, 1953) centred on the mechanism by which the streptococci introduced the amino group into the 2-position of glucose to give hyaluronate glucosamine. Reliance on whole cultures would have complicated this work owing to the complex nutritional requirements of streptococci, but washed suspensions of the organisms synthesized hyaluronate in the presence of glucose, phosphate, magnesium and simple nitrogen sources such as glutamine. Such a system was clearly much simpler than most of those hitherto used in the study of mucopolysaccharides. The earlier work (Lowther & Rogers, 1953) suggested that the amide group of glutamine was transferred to form hyaluronate glucosamine, and during the course of this work Leloir & Cardini (1953) showed that glutamine was also involved in the biosynthesis of hexosamine by extracts of *Neurospora*. The latter result raises hope that the mechanism described in this paper for the biosynthesis of hyaluronate glucosamine may have general application to the biosynthesis of hexosamine. A preliminary report of this work has already appeared (Lowther & Rogers, 1955).

METHODS

Cultivation of the organisms and preparation of suspensions

A single strain of *Streptococcus haemolyticus* (Lancefield Group A), kindly supplied by Dr R. E. O. Williams (Central Public Health Laboratory, Colindale Avenue, London, N.W. 9), called T13, has been used throughout the present work. The medium used for growth of the organism was Hedley Wright broth (Wright, 1933) containing 1% of glucose, 2·5% of sodium β-glycerophosphate and 10% of normal horse serum. The supplements were added aseptically as sterile solutions to the autoclaved broth. The organisms were grown in this medium at 35±0·5° without aeration. In order to avoid loss of the hyaluronate-producing capacity of the strain, various storage and subculture conditions were examined. The most satisfactory procedure was to dry cultures, grown for 18 hr. in the above medium, by the Stamp (1947) method and to revive these dried cultures in Hedley Wright broth containing 10% of sterile whole horse blood. The blood–broth cultures, which could be stored for several months at 0–4°, were streaked on to blood–agar plates which were incubated and the colonies were examined for purity of the strain and their mucoid character. To grow the rather large volumes of culture necessary for the present work a small volume (200–300 ml.) of the broth–glycerophosphate medium, warmed to 35°, was inoculated with 1·0 ml. of the blood–broth culture and incubated overnight at 35°. This culture was streaked on to blood–agar plates as a check on purity and added to ten times its volume of the same medium. Cell suspensions were prepared from the final culture. The age of the final culture was found to be important for the activity of the cell suspension. The harvested cells showed maximum activity when growth in the culture was equivalent to a bacterial N content of 0·033 mg./ml. Growth was measured by the optical opacity of the cultures using a Spekker absorptiometer with filters H 508 and 609. The equivalent bacterial N content was read from a curve relating optical opacity to bacterial N. The latter was determined on the organisms centrifuged from 10 ml. volumes of cultures of known opacity and washed twice with 10 ml. volumes of distilled water. When growth had reached the desired level in the bulk cultures (usually in about 4 hr.), the flasks were cooled by immersion in iced water and the cultures streaked on to blood–agar plates to check for contamination. The culture was then centrifuged and the cells washed four times with a solution containing 5 g./100 ml. of sodium β-glycerophosphate and 0·04 M-$MgSO_4,7H_2O$; all these operations were carried out at 0–4°. This repeated washing with the slightly alkaline glycero-

phosphate solution (pH 7·8) was necessary to remove as much of the hyaluronate from the surface of the organisms as possible. Finally the organisms were suspended in a solution of sodium β-glycerophosphate (5 g./100 ml.) and magnesium sulphate (0·04 M) to give an optical opacity equivalent to 1 mg. of bacterial N/ml. These suspensions were stored at 0–4° until used. Immediately before use they were centrifuged again, and the cells resuspended in the same volume of the glycerophosphate–magnesium solution.

Conditions for incubation of the suspensions. Volumes (0·5 ml.) of a solution containing 0·056 M glucose, 0·05 M phosphate buffer (Sørensen) at pH 7·0 and any other required additions were pipetted into test tubes and warmed to 37°. An equal volume of the suspension prepared above, also warmed to 37°, was then added; the final pH was 7·0. The mixtures were incubated in open test tubes for 2 hr. at 37° with gentle shaking, then cooled in iced water, centrifuged at 0–4° and the supernatant fluid was withdrawn. If estimations were not to be made immediately, these fluids were stored at −20°.

Analytical methods

The hyaluronate present in the supernatant fluids from incubated suspensions was estimated by measuring (*a*) the turbidity formed when they were mixed with acidified, diluted, normal horse serum according to Tolksdorf, McCready, McCullagh & Schwenk (1949) and (*b*) the amount of hexosamine liberated from them on acid hydrolysis.

In the turbidity method, the amount of hyaluronate, which corresponded to a given optical opacity, was interpolated from a curve relating opacity to weight of a sample of hyaluronate of known purity prepared from umbilical cord and dissolved in the fluid used for suspending the organisms. The purity of the standard sample was calculated from its hexosamine content assuming that the pure mucopolysaccharide consists of equal molecular proportions of *N*-acetylglucosamine and glucuronic acid (Meyer & Palmer, 1936). The relationship between optical opacity and concentration of hyaluronate was approximately linear over the range of 0·1–0·6 mg. Therefore, whenever possible, the supernatant fluids were diluted to contain between 0·2 and 0·5 mg. If less than 0·2 mg. of hyaluronate was available in a sample an approximate answer only could be obtained. The specificity of this method was improved by incubating critical samples with ten turbidity-reducing units (Tolksdorf *et al.* 1949; Rogers, 1953, 1954) of partially purified streptococcal hyaluronidase (Rogers, 1946) for 10 min. at 37° and pH 6·0. This enzyme is known (e.g. Chauncey, Lionetti & Lisanti, 1953) to be more specific than hyaluronidase preparations from mammalian testes. When, after this incubation, no turbidity was given on addition of the acidified serum reagent it was considered strong additional evidence in favour of the original turbidity being entirely due to the reaction between hyaluronate and the serum reagent.

The amount of hexosamine liberated by acid hydrolysis of the supernatant fluids was estimated as follows: the supernatant (0·5 ml.) fluid was mixed with 8 N-HCl (0·5 ml.) in an ampoule which was sealed and heated in a boiling-water bath for 4 hr. The HCl was then removed by repeated evaporation *in vacuo* and the amount of hexosamine was estimated by the Elson & Morgan (1933) technique; the colour formed was read with a Unicam spectrophotometer set at 535 mμ. All results are expressed as μmoles of hexosamine hydrochloride/ml. of the supernatant fluid. Sodium β-glycerophosphate, at concentrations greater than 0·5 g./100 ml. in the final test, increased colour formation caused by known amounts of glucosamine–HCl. Therefore, supernatants were always diluted so that less than this concentration of it was present.

Examination of the supernatant fluids by the Elson & Morgan (1933) method before acid hydrolysis gave negative results; thus free glucosamine was not present. Paper-chromatographic examination of the supernatant fluid with neutral phenol as solvent confirmed the negative results; no *N*-acetylglucosamine or other sugars were detected on the chromatograms, which were sprayed with Ehrlich's reagents, aniline phthalate and anisidine hydrochloride (Partridge, 1948, 1949; Hough, Jones & Wadman, 1950). It was also shown that, after quantitative precipitation of the material in the supernatant fluid with acidified normal serum, as in the ordinary turbidity method for estimating hyaluronate, no hexosamine could be detected in the acid-hydrolysed filtrate. If, however, the supernatant fluids had first been treated with streptococcal hyaluronidase all the hexosamine remained in the filtrate after the addition of acidified serum reagent. The results of these experiments are consistent with the supposition that the hexosamine liberated on acid hydrolysis has all been present as part of hyaluronic acid. The quantitative agreement, however, between the amount of hyaluronate estimated from the turbidity test and from the amount of hexosamine liberated on hydrolysis (later referred to as 'bound glucosamine') was not completely satisfactory. In the work to be described the turbidity reaction was used to obtain qualitative information about the substances affecting hyaluronate synthesis, whilst the estimation of bound glucosamine was relied upon in the more crucially quantitative aspects. The discrepancies between the two methods are probably due to factors such as the influence of different degrees of polymerization of the mucopolysaccharide upon the turbidity reaction.

Other methods of analysis used were: ammonia, as described by Conway (1940); glucuronic acid, by Ogston & Stanier (1951) and Dische (1947); and glutamic acid, by the decarboxylase of whole *Clostridium welchii* strain SR 12 cells, as described by Gale (1945). Ash was determined by heating at 600° to constant weight, and moisture by drying to constant weight at 105°. Total N was estimated either by the micro-Kjeldahl procedure, using copper selenite as catalyst, or by the method of Conway (1940), according to the amount of material available.

Samples of amino acids, including glutamine and asparagine, were checked for ninhydrin-reacting contaminants by paper chromatography.

The methionine sulphoxide was synthesized as described by McIlwain, Roper & Hughes (1948); on chromatography in neutral phenol, phenol–ammonia, and butanol in equilibrium with 1% aqueous heptanoic acid, it showed only a single spot with ninhydrin.

Identification of the hexosamine as glucosamine. The hexosamine liberated on acid hydrolysis of the supernatant fluids and of isolated hyaluronic acid was identified as glucosamine by means of: (i) its rate of travel on paper chromatograms, in neutral phenol, phenol–ammonia and butanol–heptanoic acid; (ii) its rate of travel on a column

of the ion-exchange resin Dowex-50 developed with 0·3 N-HCl (Gardell, 1953); (iii) agreement of the absorption curve of the colour given by acid hydrolysates of the supernatant fluids in the Elson & Morgan (1933) test with that given by pure glucosamine; and (iv) by isolation of the hexosamine hydrochloride by method (ii) above and crystallization from aqueous ethanol mixtures. The crystals were then examined by X-ray crystallography by Mrs O. Kennard of this Institute, to whom we are indebted for help in this matter. Plate 2 shows the picture obtained alongside that of a sample of known glucosamine hydrochloride.

RESULTS

Preliminary experiments showed that when washed suspensions of streptococci were incubated for 2 hr. at 37° with an equal volume of a 1:5 dilution of Hedley Wright broth and a final concentration of 0·5 g./100 ml. of glucose, substances were subsequently found in the supernatant fluid which gave turbidity with the acidified serum reagent (see Methods section). With four different batches of cells, the turbidity obtained was equivalent to 0·2–0·4 mg./ml. of potassium hyaluronate. When broth was omitted the values were reduced to 0·04–0·1 mg./ml. The presence or absence of glucose made little difference when broth was not present; in the presence of broth, glucose was essential for the formation of the material giving turbidity. Incubation of the supernatant fluid with streptococcal hyaluronidase abolished its turbidity-forming properties. It was, therefore, likely that the suspensions were synthesizing hyaluronate in the presence of diluted broth and glucose. No detectable increase in bacterial nitrogen occurred. The hyaluronate in the supernatant fluids from suspensions incubated with broth and glucose did not appear to arise from decapsulation of the organisms. Washed cells of strain T 13, either before or after incubation, were not visibly encapsulated when examined microscopically using the indian ink staining method, despite their ability to form large amounts of extracellular mucopolysaccharide. Likewise, if the cells were suspended in 0·1 M phosphate (Sørensen) buffer at pH 7·0 at the same density as that used for the incubation and heated at the temperature of a boiling-water bath for 10 min., no significant amounts of hyaluronate were extracted. Seastone (1939) has used this method for extracting capsular hyaluronate from streptococci. The heat treatment also extracts small amounts of substances soluble in organic solvents and resistant to the action of hyaluronidase. The turbidity given by these substances with the acidified serum reagent obscures the turbidity given by any small amounts of capsular hyaluronate which may also be extracted. It is not possible, for this reason, to be sure of the origin of the small amount of hyaluronate which appeared in the supernatant fluid when the suspensions were incubated at 37° in the absence of broth. Our interest was in analysing the stimulatory effect of broth, and since the values obtained in the absence of added nitrogen source were not usually more than 10–15 % of those in its presence, they were subtracted as a 'blank value' and their origin was not further investigated.

It has been shown (Morison, 1941; McClennan, 1954; Faber & Rosendal, 1954) that even encapsulated hyaluronate-producing strains, including T 13, can form hyaluronidase. Before proceeding with a more detailed examination of the mechanism of the effect of broth, it was necessary to ensure that sufficient hyaluronidase was not formed by the strain, under the suspension conditions used, to lower the apparent concentration of hyaluronate measured by the turbidity method. To test for the presence of hyaluronidase activity, 0·4 ml. of suspension was added to 0·5 ml. of a solution containing 1 mg. of bacterial hyaluronate. This mixture was incubated for 2 hr. at 37°. The supernatant fluid from this suspension was then analysed for hyaluronic acid content by the turbidity method. No change in concentration was found. Further test of the suspensions by the very sensitive viscosity-reducing test (McClean & Hale, 1941; McClean, 1943) showed that a very slight hyaluronidase activity was present, but it was apparently too small to affect the turbidity caused by the mucopolysaccharide although sufficient to lower viscosity appreciably in 1–2 hr. incubation. This small enzymic activity could be safely disregarded.

Incubation of the suspensions with broth and glucose for longer than 2 hr. led to no change in the amount of hyaluronate. Therefore, this period of incubation was used in all the later work.

Replacement of broth by defined nitrogen sources

In a series of experiments the broth addition to the suspensions was replaced in turn by twenty-one different amino acids, asparagine, glutamine, glucosamine and ammonium sulphate, chloride and acetate. A final concentration of 0·5 g./100 ml. of glucose was always present. All these compounds were tested at a final concentration equivalent to 10 μg. atoms of nitrogen/ml. After incubation under the usual conditions the supernatant fluids were assayed for hyaluronic acid by the turbidity method. Table 1 summarizes the results obtained in five such experiments. It will be seen that glutamine allowed the synthesis of the largest amounts of hyaluronate in each of the five experiments. Ammonium salts, serine and asparagine were active with some batches of cells, but not with others. The remainder of the compou ds tested were always inactive.

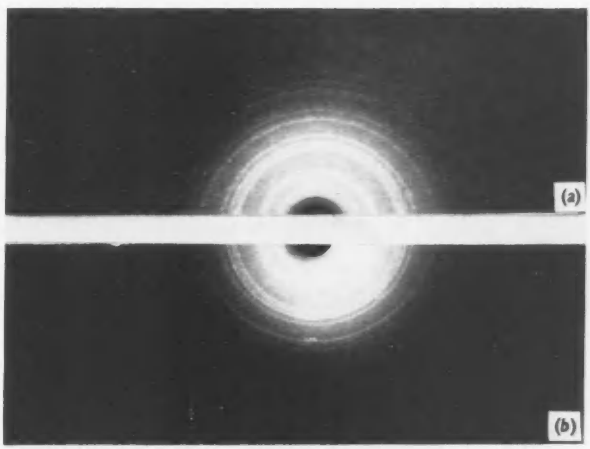

Plate 1. X-ray powder diagram of bacterial hyaluronate glucosamine (*a*) compared with that of an authentic sample of glucosamine isolated from chitin (*b*).

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(*Facing p.* 306)

Further examination of the relationship between ammonium salts and the synthesis of hyaluronate showed that, if the cell suspensions were examined within 24 hr. of preparation, they were active with the inorganic nitrogen source. This ability was lost as the suspensions stood at 0–4°, but could be partially restored by the addition to the system of the supernatant fluid from the stored suspension. The cells used for the experiments recorded in Table 1 had always been first centrifuged and resuspended in fresh glycerophosphate solutions. It thus seemed likely that some substance essential for the activity of the suspensions, when only storage, yet synthesis in the presence of L-glutamine remained unaffected. The addition of L-glutamate, together with ammonium ions, completely restored the ability of the aged preparations to use inorganic nitrogen. This phenomenon has been examined in a considerable number of experiments, always with similar results, but the following variations from the pattern in Table 2 have been noted. (1) Ammonium ions are not always as effective as glutamine for suspensions 24 hr. old. (2) The amount of hyaluronate synthesized in the presence of glutamine also sometimes falls during ageing. These phenomena have not been examined.

Table 1. *Influence of nitrogen-containing substances upon the synthesis of hyaluronate by streptococcal suspensions*

The suspensions were prepared as under Methods and contained final concentrations of 0·5 g./100 ml. of glucose, 0·02 M-$MgCl_2$ and sufficient of the nitrogen source to give a final concentration of 10 μg. atoms of N/ml. Incubation was for 2 hr. at 37°.

Nitrogen source	Hyaluronate (mg./ml.)				
Expt. no.	1	2	3	4	5
NH_4^+ (as sulphate, chloride or acetate)	0·27	—	0·38	0·32	—
Glucosamine–HCl	0·09	—	—	—	—
Amino acids* *A*†	approx. 0·04	approx. 0·04	—	—	—
B‡	—	—	—	—	0·04
Serine	0·27	—	—	—	0·04
Asparagine	0·27	0·17	approx. 0·03	0·33	approx. 0·03
Glutamine	0·54	0·40	0·48	0·61	0·37

— Not examined.

* Amino acids tested separately.

† *A*: glutamic acid, proline, hydroxyproline, threonine, alanine, methionine, tyrosine, glycine, cystine.

‡ *B*: aspartic acid, lysine, cysteine, leucine, norleucine, isoleucine, valine, histidine, ornithine, citrulline, phenylalanine.

ammonium ions and glucose were present, was leaking from the cells during the storage period. Glutamine was consistently active with both fresh and aged preparations of cells, whilst L-glutamic acid was never active. Glutamic acid might have been lost from the cells during standing, and fresh cells with an adequate internal pool of the amino acid might be able to synthesize glutamine from the added ammonium ions, whereas aged preparations of cells from which some glutamate might have leaked would be unable to do so. To test this supposition, a suspension 24 hr. after preparation, and a suspension of these same cells after standing for 4 days in the usual sodium β-glycerophosphate solution, were centrifuged and resuspended in fresh solution. Three duplicate 0·5 ml. volumes of the suspensions were incubated with (*a*) ammonium chloride, (*b*) ammonium chloride together with an equimolar concentration of L-glutamate, and (*c*) L-glutamine. The final concentration of ammonium chloride in (*a*) and (*b*) was 5 μmoles/ml. as was that of L-glutamine in (*c*). Ammonium ions alone were as effective for the fresh suspension as was L-glutamine (see Table 2). No hyaluronate, however, was synthesized by the preparation from inorganic nitrogen alone after a further 3 days

Table 2. *Effect of storing suspensions of streptococci at 0–4° upon the synthesis of hyaluronate in the presence of different nitrogen sources*

The suspensions all contained a final concentration of 0·5 g. of glucose/100 ml. and 0·02 M-$MgCl_2$. The nitrogen sources were present at a concentration of 5 μmoles/ml. Incubation was at 37° for 2 hr.

Nitrogen source	Hyaluronate synthesized (μMG/ml.*) with suspension aged	
	1 day	4 days
L-Glutamine	0·28	0·27
Ammonium L-glutamate	0·35	0·27
Ammonium chloride	0·31	0

* μmoles of glucosamine/ml. liberated on acid hydrolysis.

In other experiments the fluid in which the cells had stood at 0–4° for several days was added to suspensions made in fresh glycerophosphate solution. This addition was partially able to restore the ability of the cells to synthesize hyaluronate in the presence of ammonium ions alone. Thus the activity of some of the original preparations of cells and not others towards inorganic nitrogen seemed to be reasonably explained. Serine, asparagine and

arginine were also sometimes active, and it seemed possible that ammonia was being liberated from these compounds. If this were so, then the addition of glutamate together with serine, asparagine or arginine should lead to the synthesis of hyaluronate by preparations of the cells which were inactive when tested with the amino acids alone.

Table 3. *Influence of adding glutamate together with asparagine and serine upon hyaluronate synthesis by streptococcal suspensions*

The concentrations of each of the amino acids was 5 μmoles/ml. The cells had stood in suspension for 4 days at 0–4° before use. Incubation was at 37° for 2 hr.

	Hyaluronate synthesis			
	Expt. 1		Expt. 2	
Additions	μMG/ml.*	mgH/ml.†	μMG/ml.*	mgH/ml.†
L-Asparagine	0·018	0	0·06	0·02
L-Asparagine + L-glutamate	0·70	0·36	0·59	0·26
L-Serine	0	0	0·06	0·01
L-Serine + L-glutamate	0·50	—	0·78	0·30
Ammonium chloride	0·01	0·03	0·06	0·01
Ammonium chloride + L-glutamate	0·60	0·31	0·52	0·40

* μmoles of glucosamine hydrochloride/ml. liberated on acid hydrolysis.
† mg. of hyaluronate/ml. as measured by the turbidity reaction.

Table 3 summarizes the results obtained when suspensions which had been stored for 4 days at 0–4° were incubated with two of these compounds at a final concentration of 5 μmoles/ml., in the presence and absence of 5 μmoles/ml. of L-glutamate. Arginine behaved in a similar manner when tested with glutamate. The supernatant fluids were examined for the concentration of hyaluronate by both the turbidity reaction and by the estimation of bound glucosamine. Both serine and asparagine behave similarly to ammonium ions and allow synthesis of hyaluronate only in the presence of L-glutamate. Previous examination (Hills, 1940) of the deaminases of haemolytic streptococci had revealed a powerful arginine dehydrolase, but not a serine or asparagine deaminase, nor, for the latter compound, a deamidase. Examination of both aged and fresh suspensions, however, showed (Table 4) that in the presence of glucose, but not in its absence, ammonia was formed from these compounds by our strain of organisms. These results are similar to those obtained for the deamidation of glutamine by McIlwain *et al.* (1948). Examination by paper chromatography of the supernatant fluid from suspensions incubated with glucose and asparagine revealed the presence of aspartic acid. Thus the organisms had deamidated the amide.

Confirmation of the synthesis of hyaluronate by the suspensions

At this point in the work, net synthesis by the suspensions of a compound with properties similar to those of hyaluronate from other sources was confirmed. Proof of this was obtained in two ways: (1) by demonstrating that a total net increase in the amount of bound glucosamine occurs when the suspensions are incubated in the presence of simple nitrogen sources; and (2) by isolating and characterizing the hyaluronate from the supernatant fluid of the suspensions.

Table 4. *Liberation of ammonia from serine and asparagine by streptococcal suspensions in the presence and absence of glucose*

The concentration of the nitrogen compounds was 5 μmoles/ml. and (when added) that of glucose was 0·5 g./100 ml. Other conditions of the suspension were as for the study of the synthesis of hyaluronate. Incubation was for 2 hr. at 37°.

		NH_3 liberated (μmole/ml.) with preparation aged	
Nitrogen compound	Glucose	1 day	4 days
Serine	–	0·17	0·10
Serine	+	0·83	0·84
Asparagine	–	0·13	0·14
Asparagine	+	0·92	0·37

– No glucose present. + Glucose present.

An increase in bound glucosamine. Volumes of a cell suspension (24 hr. old) were incubated with a final concentration of 5 μmoles/ml. of L-glutamine, ammonium L-glutamate or ammonium chloride under the usual conditions. Samples of the original suspension before and after incubation, without added nitrogen source, were included as controls. The cells were removed by centrifuging and washed three times with 0·1 M (Sørensen) phosphate buffer pH 7·0, and samples were hydrolysed with 4 N-HCl as in the ordinary method for the estimation of bound glucosamine. Samples of the supernatant fluids were also hydrolysed. Table 5 shows results obtained. It will be seen that in the presence of both glutamine and ammonium

glutamate, there is a large increase in the amount of hexosamine present in the suspensions. There is also a significant increase in the presence of ammonium chloride, but on incubation in the absence of nitrogen source there is no significant change in the hexosamine content. Thus the added nitrogen sources lead to a net synthesis of hexosamine, and examination of Table 5 shows that the whole of this increase takes place in the supernatant fluid of the suspensions. The hexosamine content of the cells does not increase, but shows a very slight fall of dubious significance.

Table 5. *Effect of incubating cell suspensions with nitrogen sources upon the total amount of hexosamine in the suspension*

The conditions of incubation of the suspensions (24 hr. old) were as before. The nitrogen sources were present at a final concentration of 5 μmoles/ml.

	Hexosamine (μmoles/ml. of suspension)		
Nitrogen source	In cells	In supernatant fluid	Total
None (cells before incubation)	0·77	0	0·77
None (cells after incubation)	0·64	0·35	0·99
L-Glutamine	0·67	1·86	2·53
Ammonium L-glutamate	0·58	1·40	1·98
Ammonium chloride	0·58	0·79	1·37

Table 6. *Analysis of samples of streptococcal hyaluronic acid*

The samples were prepared as described in the text. The stated values have been corrected for the ash content of the samples, For this purpose only the metal ion of the ash (supposed as K_2CO_3 or Na_2CO_3) was taken into account.

		Hexosamine		Glucuronic acid		Total N		Optical rotation
Sample	Ash (%)	(%)	Mol.prop.*	(%)	Mol.prop.*	(%)	Mol.prop.*	$[\alpha]_D$
P 17_1	3·9	35·5	0·78	36·0	0·74	4·57	1·28	−116·5°
P 17_{1-V}	10·2	39·1	0·88	44·0†	0·94	2·81	0·80	−97°
				45·2‡	0·97			

* Molecular proportion of substance per repeating unit of *N*-acetylglucosamine and glucuronic acid.
† Determined by the method of Ogston & Stanier (1951).
‡ Determined by the method of Dische (1947).

Isolation of hyaluronate. A large volume of suspension (100 ml.) was incubated in the presence of 5 μmoles/ml. of ammonium L-glutamate and 0·5 g./100 ml. of glucose for 2 hr. at 37° and the organisms were removed by centrifuging. The supernatant fluid was then placed in supported cellophan tubing and the pressure inside the tube was raised to 60 cm. Hg. Distilled water (6 l.) flowed around the outside of the cellophan during 48 hr. at 0–4°. This process both removed the salt and concentrated the mucopolysaccharide solution. The dialysed concentrated contents of the tube were then centrifuged to remove a small amount of insoluble material and the solution was dried from the frozen state. Remaining moisture was removed from the powder by heating at 80° and 15 mm. pressure over P_2O_5 followed by storage at room temperature over P_2O_5 at 0·1 mm. pressure. This drying procedure was repeated on all samples immediately before analysis. The dry powder was extracted successively with absolute ethanol, chloroform and ether, each for 1 hr. in a Soxhlet apparatus. This extraction led to a removal of a lipid material which formed 10–20 % of the weight of the sample. The polysaccharide analyses recorded in Table 6 show the composition of lipid-free material. Both these samples were converted into the free acid as follows: 10 mg. were dissolved in water (3 ml.) and shaken with 400 mg. of Dowex-50 (acid form, previously washed with water until the washing water was between pH 5·0 and 7·0). The resin was removed by filtration and the free hyaluronic acid (7 mg.) was dried from solution as before. Analysis of this product is given opposite P 17_1 in Table 6.

Hyaluronate prepared in this way, but not treated with resin to remove salts, was then fractionated with ethanol as follows: 100 mg. were dissolved in 20 ml. of distilled water and chilled to 0°. Ethanol (46 ml.) containing 10 % of potassium acetate, chilled to −10° and at pH 9·0, was added and the mixture was allowed to stand for 30 min. at −10°. The precipitate was collected, washed three times with 10 ml. volumes of ethanol, dried and redissolved in 10 ml. distilled water. This precipitation was repeated three times. Finally, the purified product was converted into the free acid by passing a solution of it through a Dowex-50 column

(5 × 1 cm.). The effluent solution was pressure-dialysed and dried for analysis. In Table 6 this sample is called $P17_{1-V}$.

Examination of the ultraviolet absorption spectrum of a solution of the sample $P17_1$ showed no specific absorption at 260 mμ., indicating the absence of nucleic acid, but showed an inflexion at 280 mμ. Therefore the high nitrogen content of the sample before fractionation suggests that the hyaluronic acid is contaminated by about 10% of its weight of protein. This suggestion is supported by the presence in acid hydrolysates of a limited number of substances reacting with ninhydrin. The rate at which these substances travelled down a chromatograph paper in a butanol–acetic acid solvent system suggested that glutamic acid, arginine and alanine were the chief constituents. This small protein contamination was removed by alkaline ethanol fractionation and 93% by weight of the purified material was accounted for by *N*-acetylglucosamine and glucuronic acid. It is noteworthy, however, that the optical rotation of −97° for the purified material remains greater than values previously found for bacterial hyaluronate (i.e. −67·5° by Topper & Lipton, 1953, and by Roseman *et al.* 1953, and −53° to −86° by Kendall *et al.* 1937), or for mammalian hyaluronate, the accepted value for which is about −76°.

An infrared spectrum of the unfractionated material was kindly made for us by Dr S. F. D. Orr of the Chester Beatty Research Institute. The result he obtained agreed well with his previously published figure (Orr, 1954) for mammalian hyaluronate, although the ratio of absorption at the $-CO_2H$ position to that at the $-CO.NH_2$ position was 1·10 compared with 1·00 for the mammalian umbilical cord preparation. An electrophoretic examination was also made of an 0·2% solution of the unfractionated hyaluronate in buffers of pH 5·0 and 7·0 and ionic strength 0·1 μ. Only single peaks were observed in both buffers during a 1·5 hr. run. The peaks were not, however, completely symmetrical.

Relationship between glutamine synthesis and hyaluronate formation

Glutamine and ammonium L-glutamate were added to suspensions to give final concentrations of from 0·25 μmole up to 1·0 μmole/ml. and after incubation under the usual conditions the amount of hyaluronate present in the supernatant fluids was measured, by both the turbidity reaction and the amount of bound glucosamine present. It will be seen from Fig. 1 that, between these limits, the amount of hyaluronate synthesized is directly proportional to the concentration of either of the compounds added. In other experiments, in which more than 1 μmole/ml. of either glutamine or ammonium L-glutamate was added, no more mucopolysaccharide was synthesized. It may be noted that the plots of the values for bound glucosamine (expressed as mg./ml.) and of the values for hyaluronate estimated by the turbidity reaction are exactly parallel: this provides further evidence that both methods measured the concentration of the same substance, hyaluronate.

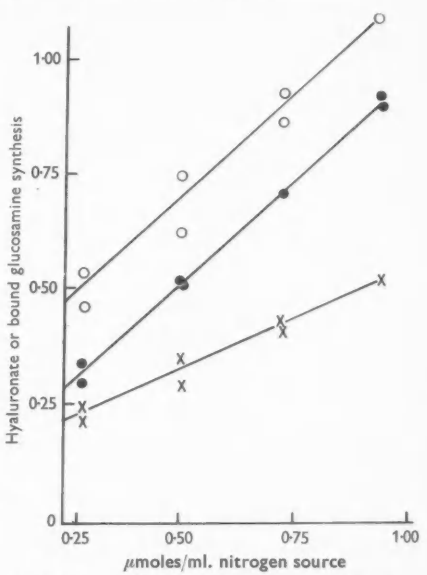

Fig. 1. Influence of glutamine and ammonium glutamate concentration on the synthesis of hyaluronate (measured by the turbidity reaction) and of bound glucosamine by suspensions of streptococci. Conditions of incubation as under Methods. ●, mg./ml. hyaluronate as measured by the turbidity reaction; ○, mg./ml. × 10^{-1} bound glucosamine in supernatant fluid; ×, μmoles/ml. bound glucosamine in supernatant fluid.

More detailed examination of the relationship between the uptake of nitrogen by the cells and the synthesis of hyaluronate was undertaken. Volumes of suspension (16 ml.) were incubated under the usual conditions, with ammonium glutamate (0·74–1·25 μmoles/ml.) and glutamine (1·25 μmoles/ml.). The concentrations of ammonia, glutamate and bound glucosamine were then estimated in the supernatant fluids. The results obtained are shown in Table 7, where it will be seen that approximately equal molecular proportions of ammonia and glutamate disappear from the solution during incubation. Concomitantly, from 0·48 to 0·89 mole of hyaluronate glucosamine appears per mole of ammonia disappearing; in five out of the eight

experiments recorded this ratio was between 0·85 and 0·89. If the concentration of the nitrogen source was raised above 1·25 μmoles/ml., less than this molecular proportion of bound glucosamine always appeared. These results seemed to be most simply explained by supposing that, with the lower concentrations of available nitrogen, about half was converted into hexosamine. It had to be assumed in the experiments yielding smaller amide. The influence of methionine sulphoxide (α-amino-γ-methylsulphinylbutyric acid) in the system was examined. Methionine sulphoxide, as is well known (Elliott & Gale, 1948; McIlwain *et al.* 1948), inhibits the synthesis of glutamine from ammonium glutamate by a variety of both mammalian and bacterial systems. Various concentrations of this substance were added to the usual system containing either glutamine or ammonium

Table 7. *Relation of the uptake of ammonia and glutamate by cells to the production of hyaluronate glucosamine*

Cell suspensions were incubated for 2 hr. at 37° with the stated amounts of the nitrogen sources, and the concentrations of the various substances in the supernatant fluids were measured. Each experiment was performed with a different batch of cells.

Experiment	Nitrogen source	Concn. (μmoles/ml.)	Hyaluronate glucosamine (μmoles/ml.)	Loss from supernatant fluid: Ammonia (μmoles/ml.)	Loss from supernatant fluid: Glutamate (μmoles/ml.)
1	L-Glutamine	1·25	0·33	0·68	0·59
	Ammonium L-glutamate	1·25	0·35	0·68	0·73
		1·25	0·70	0·59	0·68
		0·74	0·42	0·47	0·49
2	L-Glutamine	1·25	0·70	0·81*	—
		1·25	0·70	0·78*	—
	Ammonium L-glutamate	1·25	0·70	0·84	—
3	Ammonium chloride	1·25	0·33	0·57	—

* These figures represent disappearance of amide nitrogen from the supernatant fluid as estimated by the method of Krebs (1948).

Table 8. *Influence of α-amino-γ-methylsulphinylbutyric acid (methionine sulphoxide) on the synthesis of hyaluronate glucosamine*

The suspensions were prepared in the usual manner, were mixed with the appropriate concentrations of nitrogen source and methionine sulphoxide and incubated 2 hr. at 37°. The concentrations of the various substances were measured in the supernatant fluid after centrifuging. The concentration of glucose was 0·5 g./100 ml.

Nitrogen source	Concn. (μmoles/ml.)	Methionine sulphoxide concn. (μmoles/ml.)	Remaining in supernatant fluid: NH_4^+ (μmoles/ml.)	Remaining in supernatant fluid: Glutamate (μmoles/ml.)	Hyaluronate glucosamine (μmoles/ml.)	Inhibition of glucosamine synthesis (%)
L-Glutamine	1·25	0	0·47	0·45	0·42	—
	1·25	1·25	0·45	0·50	0·42	0
Ammonium L-glutamate	1·25	0	0·66	0·54	0·70	—
	1·25	1·25	1·22	0·81	0·14	80

amounts of hexosamine that, despite the low concentration of nitrogen available, some other factor limited hyaluronate synthesis. Since ammonium glutamate and glutamine behaved in all ways identically, it seemed possible that glutamine was an intermediate in the process of hexosamine synthesis, and that only the amide or the α-nitrogen atom was used.

If glutamine is a necessary intermediate between ammonium glutamate and hexosamine, synthesis in the presence of the ammonium salt should be sensitive to substances inhibiting synthesis of the L-glutamate, and the amount of bound hexosamine synthesized was measured. The amount of ammonia, glutamine and glutamate remaining at the end of the incubation period was also measured. Suitable control suspensions, containing, in addition to the usual constituents, either methionine sulphoxide only or one of the two nitrogen sources, were also incubated. The types of results obtained are illustrated in Table 8. A molecular ratio of methionine sulphoxide to nitrogen source of 1:1 inhibits the synthesis of bound hexosamine from ammonium glutamate by 80%, but has no effect

when glutamine is used. Likewise, the disappearance of ammonia in the presence of the ammonium salt, is inhibited by 95% by methionine sulphoxide. In other unquoted experiments some inhibition was recorded in the presence of glutamine, even with the ratio of methionine sulphoxide to nitrogen source of one. If this ratio was increased above one, considerable inhibition of synthesis in the presence of glutamine occurred and with a ratio of 3·0 it amounted to 74%. Synthesis from ammonium glutamate, however, was always very much more sensitive than from glutamine. These results very strongly support the intermediary role of glutamine in the biosynthesis of hyaluronate glucosamine.

Incorporation of ^{15}N into bound hexosamine

So far no available evidence suggested which of the two possible nitrogen atoms in glutamine was incorporated into the hexosamine. Moreover, evidence for the incorporation of nitrogen from the added compounds was still indirect. If it were to be shown that isotopically labelled ammonia in the presence of unlabelled glutamate was incorporated into the glucosamine of hyaluronate without considerable dilution, both of these questions might be settled. If the α-amino group of the intermediary glutamine were used, dilution of the labelled ammonia would be expected from the α-amino group of the added unlabelled glutamate.

Two experiments with [^{15}N]ammonium L-glutamate as nitrogen source were carried out. Both the cell suspensions were incubated under the usual conditions in the presence of 1·25 μmoles/ml. of ammonium L-glutamate in which nitrogen of the NH_4^+ contained 30% excess of ^{15}N. In one experiment the suspension was examined within 24 hr. of harvesting. In the other, the cells stood at 0–4° in the usual sodium β-glycerophosphate solution until no synthesis of hyaluronate occurred in the presence of ammonium ions alone. After incubation, the cells were removed by centrifuging and the hyaluronate was isolated from the supernatant fluid as described previously. The isolated mucopolysaccharide was hydrolysed for 4 hr. with 4N-HCl at the temperature of a boiling-water bath and the glucosamine was isolated from the vacuum-dried hydrolysate, using a column of Dowex-50 (Gardell, 1953). The hexosamine was wet-ashed with concentrated 'Microanalytical Standard' sulphuric acid. The ammonia was distilled from this solution into 5 ml. of 0·05N-HCl by the technique of Rittenberg (1946). The ammonia content of the solution was measured on a sample of the distillate. The remainder was then evaporated to dryness *in vacuo*, redissolved, and the nitrogen content of the sample was adjusted with unlabelled NH_4Cl to contain 1 mg./ml. of nitrogen. The excess of ^{15}N in the sample was estimated with the mass-spectrometer by Mr C. Dickinson of this Institute, for whose help we are grateful. The results of the two experiments are summarized in Table 9. It will be seen that in Expt. 1, in which freshly prepared cells were used, the isotopically labelled ammonia was diluted to the extent of 20%. In the second experiment, no dilution at all occurred, although it must be noted that the correction for non-synthesized hyaluronate was abnormally high in this experiment. In neither, however, is the dilution sufficient to suggest that the unlabelled α-amino group of the glutamate is the important nitrogen donor. Thus glutamine is synthesized from ammonium glutamate and the amide group is likely to be incorporated into the hexosamine of hyaluronic acid.

Table 9. *Incorporation of ^{15}N into hyaluronate glucosamine*

Cells were grown and suspensions prepared in the usual way, and incubation was carried out in the presence of 1·25 μmoles/ml. of ammonium L-glutamate, containing 30% excess ^{15}N in the ammonium ions. In the first experiment the cells were examined within 24 hr. of preparing the suspension. In the second the cells were stored until no synthesis in the presence of NH_4Cl alone took place.

	Expt. no. 1	Expt. no. 2
Total nitrogen of hyaluronate hexosamine isolated	0·36 mg.	0·56 mg.
Amount of non-isotopic nitrogen (as NH_4Cl) used for dilution	1·095 mg.	0·56 mg.
Isotopic excess in the diluted sample	5·86%	10·32%
Therefore isotopic excess in hyaluronate glucosamine nitrogen	23·8%	20·6%
Correction factor for unlabelled hyaluronate present as blank*	1·04	1·47
Corrected isotope excess in the synthesized hyaluronate glucosamine	24·3%	30·4%
Incorporation of ^{15}N into hyaluronate glucosamine	80%	101%

* This represents the amount of hyaluronate glucosamine nitrogen present in supernatant fluids of control suspensions, incubated in the absence of nitrogen source. It is calculated from the glucosamine, estimated by the method of Elson & Morgan (1933), in acid-hydrolysed supernatant fluids. This glucosamine was not isolated.

Synthesis of bound glucosamine by cell-free preparations

Work with cell-free preparations of *Streptococcus haemolyticus*, strain T13, is only of a preliminary nature and no evidence has yet been obtained to show whether the hexosamine synthesized is part of a larger molecule. It is included here as a final demonstration that glutamine is a necessary intermediate in the synthesis of hexosamine, thus eliminating the possibility that formation of the amide was simply necessary to transport ammonia nitrogen into the cell.

The extracts were made from suspensions prepared in the usual way, excepting that they were washed three times with 0·1 M Sørensen phosphate buffer at pH 7·0, instead of with glycerophosphate, and stored for the minimum time at 0–4°. The cells were crushed by means of the Hughes (1951) press working at −20° and the cell mush was centrifuged at 0° and 11 000 *g* for 30 min. The supernatant fluid (1 ml. volume) was then supplemented by final concentrations of 0·001 M adenosine triphosphate (ATP) as the sodium salt, 0·02 M magnesium sulphate, 0·022 M cysteine, 0·5 g./100 ml. of glucose, and 5 μmoles/ml. of either glutamine or ammonium glutamate. The final volume of the samples was adjusted to 2 ml. and they were incubated for 2 hr. at 37°. At the end of this time the samples were all hydrolysed for 4 hr. with 4 N-HCl, as in the usual estimation of hyaluronate hexosamine. The results obtained and the relative importance of some of the additions can be assessed from Table 10. It will be seen that synthesis occurred only in the presence of glutamine; ammonium glutamate was ineffective. Presumably the glutamine synthetase of the cells was not present in the extracts and in its absence glutamine is a necessary intermediate in the synthesis of hexosamine by streptococci. ATP and glucose are also essential.

Table 10. *Biosynthesis of hexosamine by cell-free extracts prepared from streptococci*

Extracts (0·5 ml.) were incubated for 2 hr. at 37° with the additions shown. Total volume in each tube was 1·0 ml. The complete system contained 5 μmoles of either ammonium L-glutamate or L-glutamine.

Nitrogen source	Omission	Glucosamine (μmoles/ml.)			
	Expt. no.	1	2	3	4
Ammonium L-glutamate	None (i.e. complete system)	0·14	—	—	0·16
	Mg^{2+}, cysteine, ATP	0·05	—	—	—
	ATP	0·03	—	—	—
L-Glutamine	None (i.e. complete system)	0·91	0·38	1·34	0·68
	Mg^{2+}	—	—	—	0·04
	Cysteine	—	—	—	0·03
	Mg^{2+}, cysteine, ATP	0·07	—	—	—
	ATP	0·08	0	0·67	0·14
	Glucose	—	0	0·10	0·03
	Glutamine	—	0·09	0·10	—

DISCUSSION

The evidence presented in this paper shows that the mucopolysaccharide synthesized by *Streptococcus haemolyticus* T13 consists of glucosamine and glucuronic acid residues and has its physical properties altered by streptococcal hyaluronidase in the same way as hyaluronate isolated from mammalian sources. The isolated mucopolysaccharide has the same infrared spectrum as mammalian hyaluronate, and approximately the same chemical composition. Ultracentrifugal (Ogston & Lowther, unpublished) and electrophoretic analysis of isolated material showed no evidence of more than one polydisperse substance. It therefore seems reasonable to suppose that we are studying the biosynthesis of hyaluronate and that all the hexosamine liberated on hydrolysis of the supernatant fluids came from this mucopolysaccharide.

The following steps are involved in the synthesis of glucosamine in this hyaluronate from ammonium ions and glucose, by the streptococcal suspensions:

$$NH_4^+ + \text{glutamate} \rightarrow \text{glutamine.}$$

$$\text{Glutamine} + \text{glucose or glucose derivative} \rightarrow \text{hyaluronate glucosamine.}$$

The work of Roseman *et al.* (1953, 1954) and Topper & Lipton (1953) shows that the carbon skeleton of the glucose derivative must be close to that of glucose itself. The results of Leloir & Cardini (1953) make it seem reasonable to suppose that this derivative will prove to be a phosphorylated hexose.

In the region of limiting nitrogen supply the conversion of the amide of L-glutamine into the amino group of glucosamine by the suspensions is remarkably efficient, approaching 100%. We have always measured the total amount of hyaluronate formed and have not concerned ourselves with the initial rates of formation. It is not, therefore,

possible to deduce to what extent hexosamine formation limits the more complicated process of mucopolysaccharide synthesis by the cell. Moreover, the proved absence of accumulation of free hexosamine or acetylhexosamine cannot help in this, since it is known that streptococci can rapidly use free amino sugars (Rogers, 1949).

It is not without interest that Reider (1953), who studied the origin of the glucosamine in ovomucoid of hens' eggs, concluded that ammonia was a much nearer precursor than amino acids and considered the possibility that glutamine might be involved but dismissed this idea. Very recently Boström, Rodén & Vestermark (1955) and Boström, Jorpes, Månsson, Rodén & Vestermark (1955) have shown that glutamine specifically stimulates the *in vitro* sulphur exchange of the chondroitin sulphate in cartilage slices.

SUMMARY

1. Washed suspensions of streptococcus T13 will synthesize hyaluronate from glucose and L-glutamine in the presence of phosphate and magnesium.

2. L-Glutamine can always be replaced by an equivalent molar concentration of ammonium L-glutamate, or by L-serine, L-arginine and L-asparagine, provided L-glutamate is also added. When freshly prepared, the cell suspensions can synthesize hyaluronate from glucose and ammonium ions alone, or from amino acids, giving rise to ammonia.

3. In the presence of ammonium L-glutamate, synthesis is much more sensitive to methionine sulphoxide than when L-glutamine is added.

4. Cell-free extracts of streptococci will synthesize glucosamine from glutamine but not from ammonium glutamate.

5. Highly efficient conversion of labelled ammonium ions into hyaluronate glucosamine is effected by streptococci in the presence of unlabelled L-glutamate.

6. Glutamine is proposed as an essential intermediate in the synthesis of hyaluronate glucosamine by *Streptococcus haemolyticus*, strain T13.

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A Study of the Conditions and Mechanism of the Diphenylamine Reaction for the Colorimetric Estimation of Deoxyribonucleic Acid

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Of the colour reactions available for the determination and identification of deoxyribonucleic acid (DNA), the reaction with diphenylamine in a mixture of acetic and sulphuric acids at 100° (Dische, 1930) has been perhaps the most widely used. The present study arose from the observation that a more intense colour was sometimes produced if, instead of being heated at 100° for 10 min., the reaction mixture was allowed to stand overnight at room temperature. As a result of this observation the procedure has been modified, principally by adding acetaldehyde to the reagents and by allowing the solution to stand for about 17 hr. at 30° instead of heating it at 100°. The modified method is 3·5 times as sensitive as Dische's original procedure, and several substances which interfere in the original method do not do so in the modified procedure.

Some observations on the mechanism of the reaction have been made; in particular it was discovered that there is a liberation of inorganic orthophosphate from DNA during the early stages of the reaction. This finding has a bearing on the structure of DNA.

The modified method has already been used in an investigation of nucleic acid metabolism during bacteriophage multiplication (Burton, 1955).

MATERIALS AND METHODS

Deoxyribonucleic acid (*DNA*). Two samples of high-molecular-weight calf-thymus DNA were used. Sample I was kindly given by Dr F. W. Putnam and had been prepared according to Hammarsten (1924). Sample II was kindly given by Dr P. W. Kent and had been prepared according to Kay, Simmons & Dounce (1952). In addition, a commercial sample of lower molecular weight (British Drug Houses Ltd.) was also used.

DNA was isolated from bacteriophage T2r which had been obtained by confluent lysis as previously described, and purified by three cycles of differential centrifuging (Burton, 1955). A portion (20 ml.) of a preparation containing 10^{11} infective bacteriophage/ml. was shaken with 5 ml. of $CHCl_3$ for 1 hr. and centrifuged, and the clear supernatant poured into 2 vol. of ethanol. After 2 hr. at −10°, the fibrous DNA was removed by a glass rod and washed with 80% (v/v) ethanol on a sintered-glass filter. The material was resuspended in 5 ml. of water and dialysed overnight against distilled water. It was re-precipitated with ethanol as before and dissolved in 5 ml. of water.

DNA was isolated from *Escherichia coli* B grown in 6 l. of mannitol medium (Burton, 1955) for 48 hr. with aeration until a thick suspension was obtained (about 1 mg. dry wt./ml.). The bacteria were harvested by centrifuging, washed with 1·5 l. of water, and suspended in 400 ml. of M-NaCl containing 0·7% of sodium dodecyl sulphate at pH 7. The suspension was agitated in the Waring Blendor for 5 min. and centrifuged. The precipitate, which contained most of the bacterial nucleic acid, was suspended in 100 ml. of M-NaCl and shaken vigorously with 100 ml. of $CHCl_3$. The layers were allowed to separate and the aqueous layer was then shaken with a further 100 ml. of $CHCl_3$. After separation, the aqueous layer was centrifuged. The slightly opalescent supernatant was poured into 200 ml. of 96% (v/v) ethanol and allowed to stand overnight at −10°. The fibrous precipitate was collected by a glass rod, washed on a sintered-glass filter with 80% (v/v) ethanol and dissolved in 11 ml. of water. At this stage the material gave an orcinol reaction (Ogur & Rosen, 1950) equivalent to 1·7 μmoles of ribose/ml., and in the diphenylamine reaction it gave a reaction equivalent to 5·8 μg. atoms of DNA-P/ml. The DNA was purified by adsorption of the ribonucleic acid (RNA) on charcoal. Norit (Harrington Bros. Ltd.) was found to be suitable and the procedure of Zamenhof & Chargaff (1951) was followed. The recovery of DNA in this step was 90%. It was precipitated with 2 vol. of ethanol as above, collected and dissolved in 12 ml. of water. This solution was centrifuged in order to remove most of the contaminating charcoal, and stored in the frozen state at −10°.

All the DNA samples were essentially free of RNA, as indicated by the orcinol reaction. The samples reacted as if they contained 0·053–0·085 mole of ribose/g. atom of DNA-P. These values are somewhat less than those recorded by previous workers (Schneider, 1945; Mayers & Spizizen, 1954). This apparent ribose content is due partly to the unspecificity of the orcinol reaction, since deoxyribose gave a reaction equivalent to 0·094 mole of ribose/mole of deoxyribose.

The calf-thymus DNA samples I and II contained no inorganic phosphate, but both the *Esch. coli* and T2 DNA samples contained 7% of their P as inorganic phosphate.

Standard DNA solutions. The highly polymerized calf-thymus DNA preparations were used. A stock solution was prepared by dissolving DNA at about 0·4 mg./ml. in 5 mM-NaOH. From this, working standards were prepared every 3 weeks by mixing a measured volume of the stock standard with an equal volume of N-$HClO_4$ and heating at 70° for 15 min. Both standards were stored in the refrigerator; no deterioration of the stock standard was detected after 6 months.

Deoxyribose, if available, is also a convenient standard. Aqueous solutions (1 mM) were stable for 3 months in the refrigerator, but after 8 months at room temperature they gave only 80–90% of the colour given by fresh solutions.

Other chemicals. 2-Deoxy-L-ribose was kindly given by Dr P. W. Kent. Acetaldehyde was redistilled and stored at 4° as an aqueous solution containing 16 mg./ml. Butyraldehyde was freshly distilled. Formaldehyde was freshly distilled into a suitable volume of water and the concentration of the resulting solution determined iodometrically. Aldol (β-hydroxybutyraldehyde) was prepared according to Claisen (1899) and distilled *in vacuo* at 60° shortly before use. DL-Glyceraldehyde was a commercial sample.

Diphenylamine reagent. This is prepared by dissolving 1·5 g. of steam-distilled diphenylamine in 100 ml. of redistilled acetic acid and adding 1·5 ml. of conc. H_2SO_4. The reagent is stored in the dark. On the day it is to be used, 0·10 ml. of aqueous acetaldehyde (16 mg./ml.) is added for each 20 ml. of reagent required. Many batches of A.R. acetic acid were found to be suitable for preparing the reagent without prior distillation, but with some samples of acetic acid the reagent developed a blue colour on standing.

Estimation of phosphorus. Inorganic orthophosphate was determined by the method of Berenblum & Chain (1938), with the slight modifications described by Bartley (1953). Total phosphorus was determined by the same method after digestion with H_2SO_4 and HNO_3 (Fiske & Subbarow, 1925).

Spectrophotometry. A Unicam SP. 600 spectrophotometer with 1 cm. cells was used.

RESULTS

Method of colour development finally adopted

In estimating nucleic acids from biological materials a suitable extract in 0·5 N-$HClO_4$ should be obtained by a method similar to that described for bacteria below. The details of preparation may have to be modified for different sources.

The nucleic acid extract is diluted with 0·5 N-$HClO_4$ so that the final solution contains between 0·02 and 0·25 μg. atom of DNA-P/ml., and a measured volume (1 or 2 ml. as convenient) is mixed with 2 vol. of diphenylamine reagent containing acetaldehyde. Tubes containing known amounts of standard DNA and a blank containing 0·5 N-$HClO_4$ but no DNA are also prepared. The colour is developed by incubating at 30° for 16–20 hr. Provided that all tubes are at the same temperature, constancy of the temperature is not essential, and variations, at least between 25° and 35°, do not appreciably affect the colour development. In the present work a constant-temperature bath at 30° was used. The optical density at 600 mμ. is measured against the blank and compared with the values obtained with the standard DNA.

Factors influencing the colour development

Aldehydes. When the reagent prepared with redistilled acetic acid was used, the optical density in the absence of added acetaldehyde was only

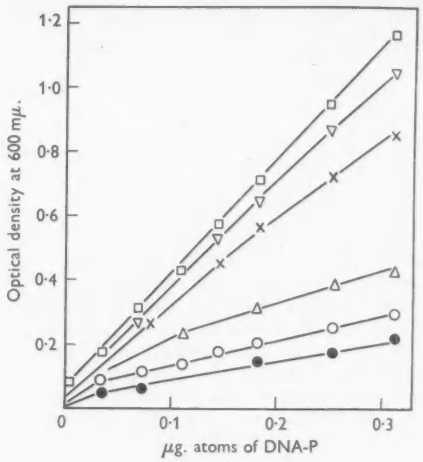

Fig. 1. Effect of acetaldehyde on the optical density obtained with different amounts of DNA. Diphenylamine reagent (2 ml.) added to 1 ml. of calf-thymus DNA (sample I dissolved in 0·5 N-$HClO_4$ by heating at 70° for 15 min.). Final concentrations of acetaldehyde (mM): ●, None; ○, 0·12; △, 0·24; ×, 0·61; ▽, 1·21; □, 1·81.

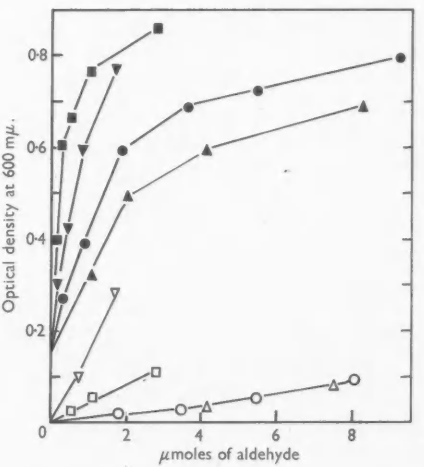

Fig. 2. Comparison of different aldehydes. Diphenylamine reagent (2 ml.) containing the aldehyde added to 1 ml. of calf-thymus DNA (sample II; 0·20 μg. atom of DNA-P) which had been dissolved in 0·5 N-$HClO_4$ by heating at 70° for 15 min. Closed symbols with DNA, open symbols no DNA: ● ○, acetaldehyde; ▼ ▽, β-hydroxybutyraldehyde; ▲ △, *n*-butyraldehyde; ■ □, glyceraldehyde.

0·15–0·3 of that obtained with the addition of acetaldehyde to the reagent. In the absence of the aldehyde the optical density for a given amount of DNA decreased with increasing DNA concentration, whereas in the presence of sufficient aldehyde the optical density is very nearly proportional to the DNA concentration. The effects of different amounts of acetaldehyde at different DNA concentrations are shown in Fig. 1. Of six aldehydes examined for their ability to replace acetaldehyde, chloral and formaldehyde had no effect. As shown in Fig. 2, DL-glyceraldehyde and DL-β-hydroxybutyraldehyde (aldol) were effective at lower concentrations than acetaldehyde, but aldol gave an appreciable colour in the absence of DNA. Higher concentrations of *n*-butyraldehyde were required. At concentrations which markedly increased the colour development from DNA, acetaldehyde and glyceraldehyde gave little colour in the absence of DNA. No effect of acetaldehyde was detected in the reaction at 100° under the conditions described by Dische (1930).

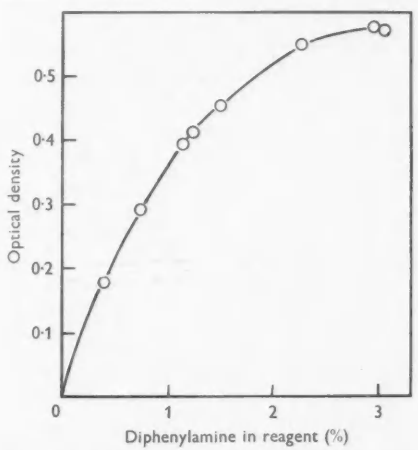

Fig. 3. Effect of diphenylamine concentration. Reagent (4 ml.) added to 2 ml. of calf-thymus DNA (sample II; 0·33 μg. atom of DNA-P) previously dissolved in 0·5 N-$HClO_4$ by heating at 70° for 15 min. The concentration of diphenylamine was varied but the amounts of H_2SO_4 (1·5%, v/v) and acetaldehyde (0·08 mg./ml.) in the reagent were kept constant.

Diphenylamine concentration. The colour intensity increased with increasing diphenylamine concentration in the range used, which was up to a concentration of 3% (w/v) in the reagent (Fig. 3). At higher concentrations diphenylamine sulphate crystallized out of the reagent. A reagent containing 3% (w/v) diphenylamine may be of value if greater sensitivity is required, but otherwise a concentration of 1·5% (w/v) is preferable, since it gives less colour in the absence of DNA.

Perchloric acid. The effect of different concentrations of the acid was investigated by using 0·25 μg. atom of DNA-P in 0·20 ml. of 0·5 N-$HClO_4$, together with 1·8 ml. of water or aqueous $HClO_4$ and 4·0 ml. of the diphenylamine reagent. The optimum concentrations of $HClO_4$ before the addition of the reagent were found to be between 0·25 and 0·65 N, and there was no detectable effect of varying the acid concentration within this range. At 0·05 N and at 1 N the colour was 92% of the maximum, but at 2 N the colour was only 70% of the maximum. The colour given by aqueous solutions of apurinic acid (Tamm, Hodes & Chargaff, 1952) or of deoxyribose in the absence of $HClO_4$ was only about 70% of that obtained with the optimum amount of $HClO_4$.

Trichloroacetic acid. This acid is likely to be present in samples taken for estimation, because in Schneider's (1945) procedure nucleic acids may be extracted by 5% trichloroacetic acid at 90°. However, if $HClO_4$ is added so as to give a concentration of 0·5 N before the addition of the diphenylamine reagent, trichloroacetic acid does not affect the final optical density, even when present at concentrations as high as 10%.

Specificity and interference. Several substances were examined for the amount of colour given with the reagent under the conditions of the DNA estimation. A solution or suspension of each substance at 4 mg./ml. in 0·5 N-$HClO_4$ was heated at 70° for 15 min. If, as with materials containing protein, a clear solution was not obtained the solution was centrifuged and the supernatant used. To 0·5 ml. of the solutions thus obtained, 0·5 ml. of 0·5 N-$HClO_4$ and 2 ml. of the reagent were added. After developing the colour in the usual way 2 mg. each of the following substances gave no detectable colour: sucrose, glucosamine, rhamnose, soluble starch, alginic acid, lactose, glucose, inositol, ascorbic acid, bovine plasma albumin, glutathione, cysteine hydrochloride, tryptophan, glycine, histidine hydrochloride, potassium gluconate, adenine sulphate, uric acid, adenosine-5′ phosphate, creatine hydrate and chloral hydrate. Under these conditions, the colour given by 4·5 $\mu\mu$g. atoms of DNA-P (i.e. 1·4 μg. of pure DNA) was readily detectable. The following substances, in 2 mg. amounts, gave a detectable colour which was less than that given by 1·4 μg. of DNA: pyruvic acid, tannic acid, fructose, indole, ribose, arabinose, xylose, α-oxoglutaric acid, acid-hydrolysed casein (Difco casamino acids), uracil. Dried human blood, glycogen and a commercial preparation of bile salts (used as an emulsion) gave readings equivalent to 6 $\mu\mu$g. atoms of DNA-P. Commercial

ribonucleic acid (5 mg.) gave a reading equivalent to 35 $\mu\mu$g. atoms of DNA-P (i.e. 11 μg. of pure DNA). Agar (2 mg.) gave a reading equivalent to 58 $\mu\mu$g. atoms of DNA-P (i.e. 18 μg. of pure DNA).

Many of the substances were also tested for their effect on the reading obtained with a standard amount of DNA. Each tube contained the substance tested, together with 0·165 μg. atom of DNA-P in 1 ml. of 0·5N-$HClO_4$ and 2 ml. of reagent. The optical density was compared with that of a blank tube containing the substance tested, but no DNA. The effect of 2 mg. of arabinose, fructose, xylose, alginic acid, adenine sulphate, uracil, glycine, histidine, chloral hydrate, α-oxoglutaric acid or manganese pyruvate was less than 2%. The presence of 2 mg. of the following substances reduced the reading: potassium gluconate (4%), acid-hydrolysed casein (10%), tryptophan (12%), ethylenediamine (9%) and diethylenetriamine (23%). Sodium chloride (20 mg.) reduced the colour by 5%. The materials found to have greatest effects were ascorbic acid, cysteine and triethylenetetramine; 0·4 mg. of ascorbic acid reduced the colour by 76%, but 0·02 mg. reduced the colour by less than 2%; 2 mg. of cysteine hydrochloride reduced the colour by 69%; 2 mg. of triethylenetetramine reduced the colour by 54%.

Comparison of these findings with those for the reaction at 100° (Dische, 1930; Cohen, 1944; Ahlström, Euler, Fischer, Halm & Högberg, 1946; Overend, 1951) shows that the modified procedure is both more specific and less susceptible to interference by the presence of other compounds.

It will be seen from Fig. 2 that both glyceraldehyde and aldol (β-hydroxybutyraldehyde) give some colour with the reagent in the absence of DNA and acetaldehyde. The colours given by these two hydroxyaldehydes are increased by the presence of acetaldehyde, but even so are much less than those obtained with equimolar amounts of deoxyribose. In the presence of acetaldehyde, 0·45 μmole of glyceraldehyde gave the same colour as 0·021 μg. atom of DNA-P, and 1·6 μmole of aldol gave the same colour as 0·13 μg. atom of DNA-P. The colour from aldol is blue and that from glyceraldehyde is yellow or, at higher concentrations, yellow-green.

Rate of colour development. It was observed that, in the early stages, the reaction mixture first appeared purple and later became blue. As will be seen from Fig. 4, the maximum of the absorption spectrum was at 560 mμ. after 1 hr. and changed to 600 mμ. after 7 hr. These changes occurred whether the reaction was performed with DNA or with deoxyribose but, as will be seen from Fig. 5, in the first 3 hr. the colour developed more rapidly with the DNA standard solution than with deoxyribose. The absorption spectra of the reaction mixtures were identical for both substances after 10 hr.

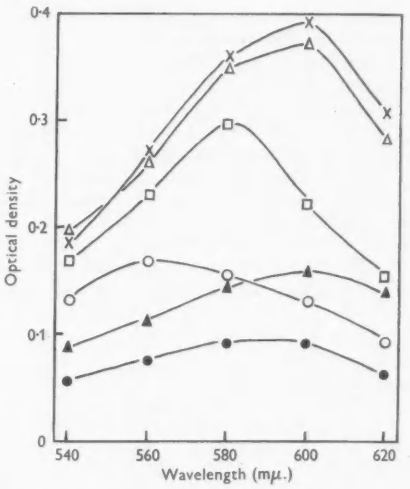

Fig. 4. Absorption spectra after different periods of colour development at 30°. Diphenylamine reagent (2 vol.) added to calf-thymus DNA (commercial material; 0·112 mg. atom of organic P/l. dissolved in 0·5N-$HClO_4$ by heating at 70° for 15 min.). Time of reaction at 30°: (*a*) with acetaldehyde (0·08 mg./ml.) added to the reagent: ○, 1 hr.; □, 2·2 hr.; △, 7 hr.; ×, 24 hr.; (*b*) no acetaldehyde: ●, 10 hr.; ▲, 24 hr.

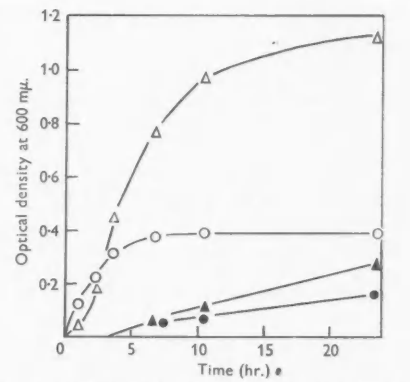

Fig. 5. Comparison of the rates of colour development with DNA and with 2-deoxy-L-ribose. DNA as for Fig. 4: ○, with acetaldehyde; ●, no acetaldehyde. Deoxyribose (0·18 mM in 0·5N-$HClO_4$) mixed with 2 vol. of reagent: △, with acetaldehyde; ▲, no acetaldehyde.

Acid treatment of the DNA. In the experiments described above the DNA was heated for 15 min. at 70° in 0·5 N-$HClO_4$ before use. This was done so that the behaviour of the DNA would be similar to that of the $HClO_4$ extracts obtained from bacteria (see below). This treatment of the DNA increased the rate of colour development (Fig. 6), but caused a small drop in the final optical density, which amounted to 7·5% after heating for 30 min. and 14% after heating for 60 min. Heating with 5% trichloroacetic acid at 90° for the same times had similar but slightly less effects on the optical densities obtained.

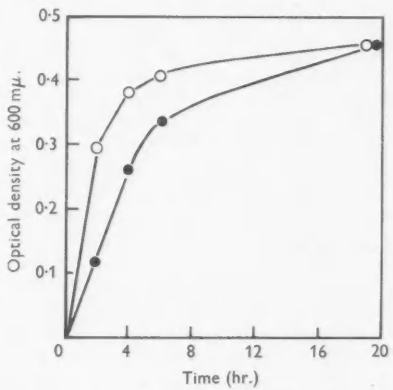

Fig. 6. Pretreatment of DNA with dilute $HClO_4$ at 70° and its effect on the rate of colour development. Reagent (2 ml.) added to 1 ml. of calf-thymus DNA (sample I, 0·135 μg. atom of DNA-P) in 0·5 N-$HClO_4$: ○, DNA heated at 70° for 15 min. in 0·5 N-$HClO_4$ before adding the reagent; ●, DNA not heated with $HClO_4$. (Although the DNA did not dissolve in the $HClO_4$ without heating, it dissolved when the reagent was added.)

Comparison of deoxyribose and DNA

The optical densities obtained with the DNA samples from three sources (calf thymus, *Esch. coli* and bacteriophage T2) were compared with those obtained from a standard solution of deoxyribose over a range of concentrations between 0·03 and 0·1 mM deoxyribose or 0·06 and 0·2 mg. atom of DNA-P/l. in the solution before addition of the reagent. For the four DNA samples (calf-thymus samples I and II, *Esch. coli*, and bacteriophage T2) the ratios of moles of apparent deoxyribose to g. atoms of organic phosphorus were respectively 0·475, 0·475, 0·485 and 0·460. For each of the DNA samples these ratios were constant within ±1·5% over the range of concentrations studied.

Liberation of inorganic phosphate

To obtain further information about the reaction between DNA and diphenylamine, the amount of inorganic orthophosphate which appeared in the reaction mixture was measured. It was found that approximately 12% of the DNA-P appeared as inorganic phosphate in 20 min. at 30° and after 150 min. between 24 and 25% of the DNA-P was liberated. This liberation of phosphate required the presence of diphenylamine but was independent of the addition of acetaldehyde. It also occurred when the acetic and sulphuric acid mixture was replaced by formic acid, but not if the mixture of acids was replaced by methanol.

The amounts of inorganic phosphate liberated from calf-thymus DNA in the presence of diphenylamine following different periods of heating in trichloroacetic acid at 90° are shown in Table 1. The table also shows the small amounts of phosphate found under similar conditions in the absence of diphenylamine. It will be seen that the amount of phosphate released in the presence of diphenylamine is not appreciably influenced by the time of the previous treatment with trichloroacetic acid.

Table 1. *Liberation of inorganic phosphate from deoxyribonucleic acid*

Calf-thymus DNA (sample II; 20·4 μg. atoms of P) was suspended in 20 ml. of water and dissolved with the aid of 12 μmoles of NaOH. Samples were heated at 90° with 0·1 vol. of 55% (w/v) trichloroacetic acid as indicated, cooled and mixed with 2 vol. of 1·5% diphenylamine dissolved in (*a*) formic acid, or (*b*) acetic acid containing 1·5% (v/v) of H_2SO_4. Control mixtures were also prepared not containing diphenylamine. Inorganic phosphate was determined after incubating at 30° for 17 hr.

Time of pretreatment at 90° (min.)	Inorganic phosphate liberated (% of total DNA-P)			
	Formic acid		Acetic acid + H_2SO_4	
	Diphenylamine	No diphenylamine	Diphenylamine	No diphenylamine
0	24·5	—	26	—
4	26·4	0·7	27·3	1·4
6·5	—	1·0	28·2	1·5
15	27·0	3·4	26·5	2·1
40	31·0	4·5	30	4·3

The method of phosphate estimation used in these experiments (Berenblum & Chain, 1938) was not interfered with by the presence of 1 ml. of the diphenylamine reagent nor by 1 ml. of 1·5% diphenylamine in formic acid. The method is based on the blue colour formed by the reduction of phosphomolybdate which has been extracted from acid solution into *iso*butanol. Although the blue colour from the diphenylamine–DNA reaction is also extracted into the *iso*butanol, it does not interfere in the phosphate estimation since it does not absorb appreciably at 750 mμ., which is the wavelength of the light which was used for measuring the optical density of the phosphomolybdate blue colour.

DNA from *Esch. coli* behaves similarly to that from calf thymus. In the presence of the diphenylamine reagent 25·5% of the organic phosphorus appeared as inorganic phosphate; only 1·1% was liberated by the acetic and sulphuric acid mixture alone.

After 17 hr. at 30° in 70% (v/v) formic acid, both fructose 1:6-diphosphate and ribonucleic acid yielded about 10% of their phosphorus as inorganic phosphate. This was not affected by the presence of 1% of diphenylamine. Under similar conditions no appreciable amount of phosphate was liberated from 3-phosphoglyceraldehyde or from glucose 6-phosphate, whether diphenylamine was present or not. It was also found that 1% aniline in 70% (v/v) formic acid can cause the liberation of phosphate from DNA, but at a slower rate than with diphenylamine.

Extraction of DNA from Escherichia coli

The diphenylamine reaction, as described in this paper, has been used to estimate DNA in *Esch. coli* (Burton, 1955). On preliminary examination, the extraction procedure which was used in this work appeared to extract 95% of the DNA. The extraction has been reinvestigated more thoroughly and now appears to extract only 90–93%, but such a difference does not affect the conclusions obtained in the previous work. By increasing the time of extraction with 0·5N-$HClO_4$ from 10 to 15 min. it is possible to increase the efficiency of extraction to 95–96%.

The procedure is based on those of Schneider (1945) and of Ogur & Rosen (1950). Each sample of the culture should contain at least 0·15 μg. atom of DNA-P with not less than 0·01 μg. atom of DNA-P/ml. of culture. Each sample is acidified with 2·5N- or 12N-$HClO_4$ to a final concentration of 0·25N and centrifuged after chilling for 30 min. The precipitate is broken up with a glass rod and stirred with 0·5 ml. 0·5N-$HClO_4$. A further 3·5 ml. of 0·5N-$HClO_4$ is added and the suspension is heated at 70° for 15 min. with occasional stirring. After centrifuging, the supernatant is decanted into a 10 ml. graduated tube. The precipitate is

Table 2. *Extraction of deoxyribonucleic acid from bacteria*

$HClO_4$ (final concentration 0·25N) was added to measured volumes of *Esch. coli* B cultures grown in a mannitol–ammonium salts medium (Burton, 1955). After cooling at 0° for 30 min. they were centrifuged and the precipitate was extracted with acid. The first two extractions (4 and 3 ml. respectively) were as indicated. The third extraction was with 1·5 ml. of 0·5N-$HClO_4$ for 15 min. at 70°. DNA was estimated on the extracts and the residue.

First 2 extractions			DNA (μμg. atom of P)					DNA in first two extracts (% of total DNA)
Conditions	Time (min.)	Vol. of culture (ml.)	1st extract	2nd extract	3rd extract	Residue	Total DNA/ml. of culture	
Expt. I								
0·5N-$HClO_4$ at 70°	10	10	418	185	43	22	66·8	90
	20	10	580	56	11	14	66·1	96
	30	10	565	54	11	14	64·4	96
N-$HClO_4$ at 70°	10	10	282	217	59	19	57·7	86
	20	10	299	185	56	19	56·1	86
5% Trichloroacetic acid at 90°	5	10	425	146	34	25	63·0	91
	10	10	533	68	17	18	63·6	95
Expt. II								
0·5N-$HClO_4$ at 70°	10	10	584	608	95	32	132	91
		6	372	354	52	22	133	91
		4	275	214	36	17	135	90
		3	216	162	21	13	137	92
	15	10	925	327	39	18	131	95
		6	587	192	27	12	136	95
		4	401	126	16	12	138	95
		3	305	92	10	8	139	95

re-extracted in the same way with a further 3 ml. of 0·5N-$HClO_4$ the two extracts are mixed and the volume is measured. A portion (1 or 2 ml., as convenient) of the extract is used for the diphenylamine reaction.

Table 2 shows the effects of some different conditions of extraction. After the extractions, as indicated, the DNA remaining in the residue was determined by suspending in a total volume of 10 ml. of 0·5N-$HClO_4$ and adding 2·0 ml. of the reagent. The material which did not dissolve remained white, although the supernatant developed the blue colour. This insoluble material was centrifuged off after the colour development and before measuring the optical density of the supernatant. It will be seen that extraction with 0·5N-$HClO_4$ was more efficient than extraction with N-$HClO_4$. Extraction with 5% trichloroacetic acid at 90° was virtually as effective as the extraction with 0·5N-$HClO_4$. The efficiency of extraction is not dependent on the amount of bacteria. The results of Table 2 also show that the amount of culture needed may be reduced by using a single extraction with 0·5N-$HClO_4$ for 20–25 min. at 70°, since under these conditions a more concentrated extract is obtained. When the DNA content of the culture is calculated from the total volume of precipitate plus extract, the value obtained is 94–95% of the total.

The precipitation of the DNA from the culture, together with the bacterial protein, appears to be complete if the culture contains 0·01–0·03 μg. atom of DNA-P/ml., since no DNA was detectable in the supernatants and also since after threefold dilution of a culture which contained 0·03 μg. atom of DNA-P/ml. the same amount of DNA was found in the precipitate as without dilution. However, after tenfold dilution of the same culture only 80% of the DNA was recovered in the precipitate. This loss was mainly the physical loss of small particles of the precipitate into the supernatant; it was prevented by adding bovine serum albumin (0·2–0·5 mg./ml. of culture) which gives a greater bulk to the precipitate but does not affect the extraction of the DNA from the precipitate. Alternatively, in estimating DNA in dilute cultures, the bacterial cells may be centrifuged before treatment with $HClO_4$.

DISCUSSION

It is already been pointed out in the previous sections that the modified procedure is both more sensitive and specific and less susceptible to interference than is the original diphenylamine reaction at 0°. The sensitivity is similar to that of the most sensitive colour reactions of DNA which have been studied. These reactions are (*a*) with indole and hydrochloric acid (Ceriotti, 1952), (*b*) with cysteine and sulphuric acid (Dische, 1930; Stumpf, 1947; Brody, 1953), (*c*) the Feulgen reaction (e.g. Dondero, Adler & Zelle, 1954) and (*d*) a reaction with *p*-nitrophenylhydrazine (Webb & Levy, 1955).

The indole and Feulgen reactions are less specific than the cysteine, the nitrophenylhydrazine or the modified diphenylamine reactions. These last three reactions do not give any appreciable colour with ribonucleic acid or non-deoxy sugars. However, the specificity and the possible interference by other compounds do not appear to have been studied as thoroughly for the reactions (*b*) and (*d*) as for the modified diphenylamine reaction. Of these three reactions the diphenylamine reaction is the most convenient to use as the cysteine–sulphuric acid reaction requires solutions of sulphuric acid so concentrated that, because of their viscosity, they are not easily pipetted with accuracy, while the *p*-nitrophenylhydrazine method involves an extraction with *n*-butyl acetate. The diphenylamine reaction may therefore be more readily adaptable for ultramicroanalysis.

Mechanism of the reaction

No colour was detected with 0·5 μmole of thymidylic acid, which is more easily hydrolysed than cytidylic acid (Manson & Lampen, 1951; Dische, 1955). In contrast to the stability of the links between pyrimidines and deoxyribose, those between purines and deoxyribose are very labile, so it appears that diphenylamine reacts with the sugar residues originally combined with purines in the DNA. The ratios of apparent deoxyribose to total phosphorus are in agreement with this mechanism and with the fact that DNA's of different origin all appear to have a purine:pyrimidine ratio very close to unity (Chargaff, 1955).

It follows from this that the inorganic phosphate liberated from DNA in the early stages of the diphenylamine reaction very probably arises from the phosphate bridges between two adjacent purine nucleotides in the polynucleotide chains. If this is so, the liberation of 25% of the total phosphorus in the DNA would be in agreement with the view that there is a random distribution of purine and pyrimidine residues along the polynucleotide chains. As discussed by Brown & Todd (1955) and by Chargaff (1955), there is at present scanty evidence about the sequence of the nucleotides in DNA, but this reaction may prove to be a useful tool when the other products of the reaction and the action of the acid alone have been studied.

The mechanism of this phosphate elimination is unknown; no similar amine-catalysed reaction has been described. However, deoxyribose 5-phosphate (Racker, 1952) and the phosphates of β-aldehydo alcohols (Meyerhof & Lohmann, 1934; Brown & Todd, 1955) are labile to acid, and possibly the

condensation of the aldehyde group with a suitable amine increases this lability.

The fact that the development of the colour is slower with deoxyribose than with acid-treated DNA indicates that free deoxyribose is not an intermediate in the diphenylamine reaction. The effect of diphenylamine on the phosphate liberation shows that the amine combines with the DNA before the phosphate is released from the sugar. Neither of these findings agrees with the views of Deriaz, Stacey, Teece & Wiggins (1949) and Overend, Shafizadeh & Stacey (1950), who consider that, in the reaction at 100°, ω-hydroxylaevulaldehyde is formed by the action of the acid on the DNA or deoxyribose and subsequently condenses with the diphenylamine. It is, of course, conceivable that condensation products (e.g. Schiff's bases) between the amine and deoxyribose or ω-hydroxylaevulaldehyde are the actual intermediates. However, for the deoxyribose derivative to be an intermediate in the reaction of DNA and diphenylamine, it would have to be formed more slowly from deoxyribose itself than from the deoxyribose residues in DNA which has been treated with 0·5N-perchloric acid for 15 min. at 70°.

The action of acetaldehyde and some other aldehydes in potentiating the colour development is obscure, but it might conceivably involve a reaction analogous to that of Doebner (1894), in which two aldehyde molecules and an aromatic amine react, with ring closure. Deoxyribose can take part in this type of reaction (Pesez, 1950), but chloral and formaldehyde do not (Velluz, Pesez & Amiard, 1948). These two aldehydes have no effect in the diphenylamine reaction.

The nature of the coloured compounds formed in the diphenylamine reaction is unknown. The changes of the absorption spectrum in the reaction at 30° show that at least two compounds are formed; these may be identical with two of the six compounds detected by Deriaz *et al.* (1949) in the reaction at 100°.

SUMMARY

1. The colour reaction of Dische (1930) between diphenylamine and deoxyribonucleic acid (DNA) has been studied and modified. The principal modifications are to add acetaldehyde and to perform the reaction for several hours at 30° instead of for 3–10 min. at 100°. The modified reaction is more sensitive and specific and less susceptible to interference by other compounds than is the reaction at 100°.

2. The colour reactions of DNA preparations from calf thymus, *Escherichia coli* and bacteriophage T2 have been compared with that of 2-deoxyribose. For the three sources the ratios of moles of apparent deoxyribose to gram atoms of DNA phosphorus were almost identical and between 0·46 and 0·485.

3. In the early stages of the diphenylamine reaction there is a liberation of inorganic orthophosphate from DNA. The amount of phosphorus thus liberated from calf-thymus DNA and from *Esch. coli* DNA is approximately 25% of the total DNA phosphorus and probably arises from the phosphate bridges between adjacent purine nucleotides in the polynucleotide chain. This value is consistent with the view that the purine and pyrimidine nucleotides are randomly distributed along the chain.

4. The conditions for the quantitative extraction of DNA from bacteria have been studied.

5. Evidence on the mechanism of the DNA–diphenylamine colour reaction is discussed and taken to indicate that neither free deoxyribose nor free ω-hydroxylaevulaldehyde are intermediates.

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The Reduction of Vinylacetate by *Clostridium kluyveri* and its Dependence on Catalytic Amounts of High-energy Acetate

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Growing cultures of *Clostridium kluyveri* ferment ethanol plus acetate, yielding mainly butyrate, hexanoate and hydrogen (Bornstein & Barker, 1948). Stadtman & Barker (1949*a–e*) and Kennedy & Barker (1951*a*) have investigated the mechanism of butyrate synthesis by this organism, using dried cells and cell-free extracts which catalyse reactions 1 and 2:

$$CH_3.CO_2H + CH_3.CO_2PO_3H_2 + 2H_2 \rightarrow CH_3.CH_2.CH_2.CO_2H + H_3PO_4 + H_2O, \quad (1)$$

$$CH_3.CH_2.CH_2.CO_2H + H_3PO_4 + O_2 \rightarrow CH_3.CO_2H + CH_3.CO_2PO_3H_2 + H_2O. \quad (2)$$

During an examination of possible intermediates, the reduction of vinylacetate (3:4-butenoate) according to reaction 3 was observed (Stadtman & Barker, 1949*e*).

$$CH_2{:}CH.CH_2.CO_2H + H_2 \rightarrow CH_3.CH_2.CH_2.CO_2H. \quad (3)$$

Several other C_4 compounds at the same oxidation level as vinylacetate were examined, including crotonate (*trans*-2:3-butenoate), *iso*crotonate (*cis*-2:3-butenoate) and 3-hydroxybutyrate; all were excluded as intermediates in reactions 1 and 2. Crotonate and *iso*crotonate were neither oxidized nor reduced by the preparations. No direct test was carried out with 3-hydroxybutyrate, but the preparations reduced acetoacetate, giving a quantitative yield of 3-hydroxybutyrate, indicating that the latter is not reduced. Vinylacetate was the only compound of those tested which was oxidized and reduced at rates compatible with the role of intermediate. In the presence of a nitrogen gas phase, vinylacetate was shown to undergo two other reactions—an anaerobic oxidation with the evolution of H_2 (reaction 4) and a dismutation (reaction 5).

$$CH_2{:}CH.CH_2.CO_2H + H_3PO_4 + H_2O \rightarrow CH_3.CO_2H + CH_3.CO_2PO_3H_2 + H_2, \quad (4)$$

$$2CH_2{:}CH.CH_2.CO_2H + H_3PO_4 + H_2O \rightarrow CH_3.CH_2.CH_2.CO_2H + CH_3.CO_2H + CH_3.CO_2PO_3H_2. \quad (5)$$

However, isotope experiments indicated that free vinylacetate was not a true intermediate in reactions 1 or 2. In the presence of unlabelled vinylacetate, [*carboxy*-^{14}C]butyrate gave rise to acetate with a higher specific activity than the vinylacetate recovered at the end of the experiment. Again, in the presence of unlabelled vinylacetate, [*carboxy*-^{14}C]acetate yielded labelled butyrate, but no isotope appeared in the residual vinylacetate.

Kennedy & Barker (1951*a*) later observed that dried cells could oxidize butyrate and vinylacetate in the absence of added phosphate, though at a reduced rate, and under these conditions acetoacetate was the main product. These observations led to the hypothesis that the C_4 compounds are oxidized and reduced in the form of complexes with a coenzyme, possibly coenzyme A (CoA) (cf. Fig. 2). This concept is supported by the observations reported in the present paper, which indicate that vinylacetate must be activated before it is reduced.

MATERIALS AND METHODS

Organism and suspensions of dried cells. Strain K1 of *Cl. kluyveri*, which was isolated by Barker & Taha (1942) and used in the studies on fatty acid synthesis by Stadtman & Barker, was used throughout.

The dried cell preparation used (Lot YE9) was made by the method of Stadtman & Barker (1949*a*), with the following modifications: (1) a slight change was made in the

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medium for large-scale cultivation described by Stadtman & Barker (1949*a*) which contains ethanol, acetate, salts and growth factors—0·05% Difco Yeast Extract replaced the biotin and *p*-aminobenzoic acid; (2) the cells were washed three times in 0·03% $Na_2S,9H_2O$ instead of twice; (3) the washed cells were freeze-dried instead of being dried *in vacuo* over $CaCl_2$ at room temperature.

Aeration inactivates the butyrate oxidizing system of *Cl. kluyveri*, and the preparations are reactivated by 1:3-dimercaptopropanol (BAL) (Kennedy & Barker, 1951*a*). The dried cells were therefore suspended in water containing 0·002M BAL; this operation was carried out anaerobically in an evacuated Thunberg tube. Samples of cell suspensions were added to manometer cups immediately before gassing with H_2.

Extract of boiled yeast (*EBY*). This was prepared by heating a 20% (w/v) suspension of '20–40' dried yeast (Fleischman, New York) at 100° for 10 min. The solid material was removed by centrifuging and the supernatant was brought to pH 8 and stored at −18° until used.

Acetyl phosphate. This was used as the dilithium salt prepared by the method of Stadtman & Lipmann (1950).

Acetyl coenzyme A. This was prepared as described by Stadtman (1952*a*) from a sample of CoA assaying 69 units/mg. (units defined by Kaplan & Lipmann, 1948). In the final chromatographic separation, the solvent used contained acetate buffer, the removal of which was essential since it inhibits the reduction of vinylacetate (see below). This was accomplished by adjusting the pH of the eluate from the chromatogram to 4–5 and passing it through a 1 × 1 cm. diam. column of charcoal. The charcoal was Nuchar C190 (West Virginia Pulp and Paper Co., Covington, Va.) and before use was treated by soaking overnight in 5N-HCl and then washing with distilled water until the pH of the eluate rose to 4–5. After application of the acetyl CoA concentrate, the column was washed with 10 ml. of water to remove the acetate, after which the absorbed acetyl CoA was eluted with 40% aqueous acetone containing 0·0165N-NH_3. The first 6 ml. of eluate was collected and evaporated to dryness under reduced pressure at room temperature. The product was used in aqueous solution, the concentration being determined as described by Stadtman (1952*a*).

Diphosphopyridine nucleotide (*DPN*). This was obtained from Dr A. Kornberg and was approximately 90% pure.

Triphosphopyridine nucleotide (*TPN*). The preparation used was made from hog liver by the method of LePage & Mueller (1949) and contained 8·7% of TPN.

Vinylacetate. The anhydrous sodium and potassium salts of vinylacetic acid were prepared from the free acid (Rietz, 1944) by neutralizing to pH 7 with the appropriate hydroxide, followed by freeze-drying (R. G. Bartsch, private communication).

When assayed by the catalytic-hydrogenation method of Harrison (1939), the hydrogen uptakes observed were 98% (Na salt) and 101% (K salt) of the theoretical values.

Reduction of vinylacetate. This was followed by using molecular H_2 as hydrogen donor and measuring the gas uptake in Warburg manometers at 26° (Stadtman & Barker, 1949*c*). The reaction was carried out at pH 8·0 in presence of KH_2PO_4–K_2HPO_4 or aminotrishydroxymethylmethane (tris) buffer. [Tris was recrystallized from ethanol: H_2SO_4 or KH_2PO_4 was added to an aqueous solution to give the requisite pH and a final tris concentration of 1·0M.]

Stadtman & Barker (1949*c*) showed that the reduction of vinylacetate by their preparation gave rise to an amount of butyrate equivalent to the H_2 uptake. An equivalence between the H_2 uptake (or butyrate production) and the amount of vinylacetate added was not shown. The formation of butyrate during the reduction of vinylacetate by the present preparation (YE9) was checked qualitatively by separating the volatile fatty acids by distillation from acid solution in the apparatus of Markham (1942). The Na salts of the acids, obtained by titration and evaporation of the distillates, were examined by paper chromatography with an ethanol–ammonia solvent as described by Kennedy & Barker (1951*b*). The results showed that the vinylacetate disappeared and butyrate appeared. No major changes in any other volatile acid were observed, although in the case of acetic acid any change would be obscured since the enzyme preparations yielded appreciable amounts of acetic acid on distillation.

The total H_2 uptakes observed varied somewhat in the range 55–70% of the theoretical quantity required for complete reduction of the added vinylacetate to butyrate. The fate of the vinylacetate not reduced to butyrate has not been established. Apart from reduction to butyrate, vinylacetate might also be removed by the anaerobic oxidation or the dismutation mentioned above (reactions 4 and 5), though these reactions were demonstrated by Stadtman & Barker (1949*c*) only in the presence of N_2. In either case, the products other than butyrate would be acetyl phosphate and acetate, and under H_2 these would be reduced to butyrate. The net result would still be an uptake of 1 mole of H_2 for each mole of vinylacetate reduced to butyrate. It therefore seems reasonable to assume that the H_2 uptake observed in the present experiments may be used as a measure of the reduction of vinylacetate to butyrate.

After a short lag period of doubtful significance, H_2 uptake followed a linear course, until it was complete (see, for example, curve *A*, Fig. 1*a*). The rates of H_2 uptake quoted were measured over the linear phase.

RESULTS

Effect of boiled-yeast extract (*EBY*) *on the reduction of vinylacetate by dried cells of* Clostridium kluyveri. When the present work was started, difficulty was experienced in obtaining preparations of dried cells which would catalyse the reduction of vinylacetate by H_2 under the conditions of Stadtman & Barker (1949*c*), although some older preparations which had been stored at −18° for up to 2 years were still highly active. Certain modifications in the method of preparation of the dried cells have been mentioned above and may account for the observed differences in behaviour.

It was found possible to activate several of the batches of dried cells prepared according to the modified method, by the addition of EBY to the test system. The results of a typical experiment are given in Table 1. This indicated that the preparation was deficient in some cofactor necessary for the reduction of vinylacetate, and attempts were therefore made to replace the EBY by various known substances.

Replacement of EBY by acetyl phosphate. The results obtained when various supplements were added to the system are given in Table 2. DPN, TPN and CoA—singly or all three together—were without effect. Neither was any activity observed when a solution of metal salts was added so as to give the concentrations obtaining in the medium used for growing the organism, nor when a small amount of ethanol was added. Difco Yeast Extract could not replace the EBY.

Table 1. *Requirement for extract of boiled yeast (EBY) for the reduction of vinylacetate by dried cells of* Clostridium kluyveri

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M-KH_2PO_4–K_2HPO_4, pH 8·0; 0·4 ml. of EBY. Na vinylacetate (17·0 μmoles) was added from the side arm after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°.

System	Rate of H_2 uptake (μl./hr.)
EBY absent, no substrate	2
EBY absent + vinylacetate	16
EBY present, no substrate	2
EBY present + vinylacetate	392

Table 2. *Tests on various substances for their ability to replace EBY in the reduction of vinylacetate*

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M-KH_2PO_4–K_2HPO_4, pH 8·0. Sodium vinylacetate (17·0 μmoles) was added from the side arm after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°. All supplements were initially present in the main compartment, except for the ethanol and acetyl phosphate, which were added from the side arm with the substrate. Metal ions were added where indicated as 0·2 ml. of a solution giving the following concentrations (μg./ml.) in the reaction mixture: $MgSO_4,7H_2O$, 200; $CaCl_2$, 10; $Na_2MoO_4,2H_2O$, 2; $MnSO_4,4H_2O$, 2; $FeSO_4,7H_2O$, 2.

Supplements	Rate of H_2 uptake (μl./hr.) Expt. 1	Expt. 2
None	6	7
EBY (0·4 ml.)	150	155
DPN (770 μg.)	7	—
TPN (170 μg.)	5	—
CoA (30 units)	12	—
DPN + TPN + CoA (amounts as above)	—	8
Difco Yeast Extract (4 mg.)	—	11
Metal ions	—	6
Ethanol (0·1 μmole)	—	8
Lithium acetyl phosphate (0·2 μmole)	—	157

However, when a small amount (0·2 μmole) of acetyl phosphate was added, hydrogen was taken up at the same rate as in the presence of EBY, suggesting that acetyl phosphate can replace the active factor in the EBY. A more detailed analysis of the effect of acetyl phosphate is shown in Table 3. Acetyl phosphate, added in the absence of vinylacetate, had no effect on the H_2 uptake, showing that the stimulation observed in presence of vinylacetate was not due to an increase in the rate of endogenous H_2 uptake.

Table 3. *Stimulation of the reduction of vinylacetate by a catalytic amount of acetyl phosphate*

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M-KH_2PO_4–K_2HPO_4, pH 8·0. Sodium vinylacetate (17·0 μmoles) and/or acetyl phosphate (0·2 μmole) were added where indicated from the side arm after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°.

System	Rate of H_2 uptake (μl./hr.)
Acetyl phosphate absent, no substrate	18
Acetyl phosphate absent + vinylacetate	22
Acetyl phosphate present, no substrate	16
Acetyl phosphate present + vinylacetate	126

Table 4. *Further observations on the effect of acetyl phosphate and EBY on the reduction of vinylacetate*

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M-KH_2PO_4–K_2HPO_4, pH 8·0. EBY (0·4 ml.) was added from the side arm or was initially present in the main compartment as indicated. Acetyl phosphate (0·1 μmole) and Na vinylacetate (10 μmoles) were added from the side arm, the former where indicated and the latter in all cases except that indicated, after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°.

System	H_2 uptake: Total volume (μl.)	Rate (μl./hr.)
No supplements	13	12
+ EBY (side arm)	105	114
+ acetyl phosphate	128	168
+ EBY (side arm) + acetyl phosphate	113	122
+ EBY (side arm), vinylacetate absent	22	10
+ hydrolysed acetyl phosphate	15	10
+ EBY (main compartment)	19	18

The stimulating action of acetyl phosphate appears to be similar to that of EBY, since when both were added to the system the total H_2 uptake was not significantly greater than with either singly (Table 4). Neither was the rate of H_2 uptake; indeed in this particular experiment the rate was significantly lower in presence of both supplements than with acetyl phosphate alone. Previously, the EBY had been placed in the main compartment of the manometer along with the *Cl. kluyveri* preparation, but in this experiment it was added from the

side arm for reasons given below. A further control (Table 4) showed that even in these changed circumstances there was no marked H_2 uptake in the absence of vinylacetate. Hydrolysis of acetyl phosphate destroyed its activating properties, indicating that the intact molecule is necessary.

The measurement of vinylacetate reduction involves a preliminary incubation before tipping in the substrate, during which hydrogen is taken up by the *Cl. kluyveri* preparation (Stadtman & Barker, 1949*e*). Stimulation of vinylacetate reduction by EBY was not obtained consistently when this supplement was preincubated with the enzyme system in the main compartment of the manometer, although in the same experiment it was active when added from the side arm along with the substrate (cf. Tables 1 and 4). This suggested that the active principle in the EBY might be unstable, and this point was examined in more detail.

Need for the simultaneous presence of vinylacetate and acetyl phosphate for reduction. In order to investigate further the stability of the activating factor, an experiment was carried out in which acetyl phosphate and vinylacetate were added separately to the system in different sequences. The results are shown in Fig. 1.

When the acetyl phosphate and vinylacetate were added simultaneously, the H_2 uptake observed was linear with time until it ceased (curve *A*, Fig. 1*a*). The total H_2 uptake observed was less than that required for the quantitative reduction of vinylacetate to butyrate but was proportional to it, since when only half the amount of vinylacetate was added the H_2 uptake was approximately halved (curve *B*, Fig. 1*a*). The significance of the blank H_2 uptake in the absence of both vinylacetate and acetyl phosphate (curve *C*, Fig. 1*a*) is difficult to assess. A slow but prolonged H_2 uptake was observed, whereas, in the manometers where vinylacetate was reduced, no such slow uptake was found after the rapid uptake due to the substrate had ceased. When the acetyl phosphate was added at zero time and the vinylacetate added 90 min. later, no H_2 was taken up (curve *D*, Fig. 1*a*), but when the times of addition of these two compounds were interchanged, H_2 was taken up at the same rate and the total uptake was approximately the same as when both components were added at zero time (curve *E*, Fig. 1*a*). It follows that whereas vinylacetate added alone is relatively stable in this system, the acetyl phosphate (or activating compound derived from it) is not.

Once the H_2 uptake due to the addition of substrate plus activator had ceased, addition of either acetyl phosphate or vinylacetate had no effect (compare curves *B*, *F*, *G*, Fig. 1*b*). On the other hand, addition of both led to a further H_2 uptake

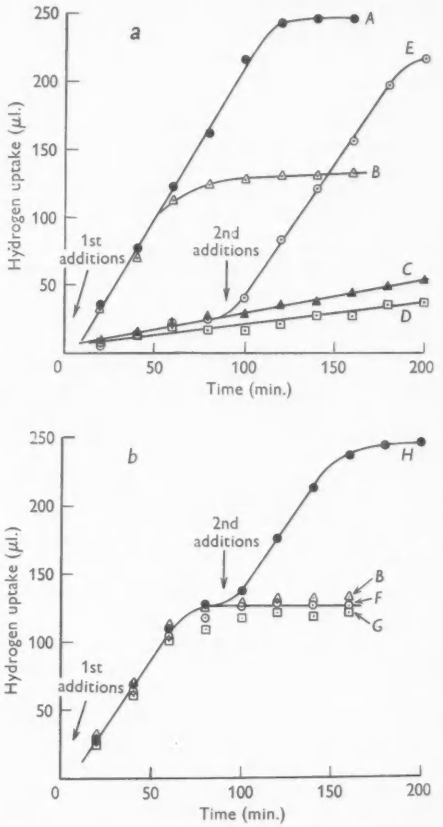

Fig. 1. Dependence of hydrogen uptake on the simultaneous presence of vinylacetate and acetyl phosphate. Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M-KH_2PO_4–K_2HPO_4; pH 8·0. Acetyl phosphate (0·2 μ-mole) and sodium vinylacetate (10 or 20 μmoles) were added from the side arm as indicated below, after saturation of the enzyme system with H_2. Figs. 1*a* and 1*b* are drawn from data obtained in a single experiment. Curve *A*: acetyl phosphate and vinylacetate (20 μmoles) added at zero time. Curve *B*: acetyl phosphate and vinylacetate (10 μmoles) added at zero time. Curve *C*: no additions. Curve *D*: acetyl phosphate added at zero time and vinylacetate (20 μmoles) at 90 min. Curve *E*: vinylacetate (20 μmoles) added at zero time and acetyl phosphate at 90 min. Curve *F*: acetyl phosphate and vinylacetate (10 μmoles) added at zero time and additional acetyl phosphate at 90 min. Curve *G*: acetyl phosphate and vinylacetate (10 μmoles) added at zero time and additional vinylacetate (10 μmoles) at 90 min. Curve *H*: acetyl phosphate and vinylacetate (10 μmoles) added at zero time and again at 90 min.

proportional to the amount of vinylacetate added (curve *H*, Fig. 1*b*). The fact that the second addition of a small amount of acetyl phosphate was without effect suggests that the uptake of H_2 ceases when the vinylacetate is exhausted. It will be noticed, however, that the total H_2 uptake was equivalent to only 55% of the vinylacetate added.

Since the second addition of vinylacetate was not reduced in the absence of extra acetyl phosphate, it follows that the activating principle must have been destroyed during the interval between but is stabilized during the metabolism of vinylacetate.

Influence of the amount of acetyl phosphate on the reduction. It seemed possible that there might be a threshold concentration of acetyl phosphate below which the rate of reduction would be dependent on the concentration of this factor. However, experiment showed that, if reduction occurred at all, both the rates and total H_2 uptake in each experiment were approximately equal, whatever the amount of acetyl phosphate added (Table 5). No

Table 5. *Effect of the amount of added acetyl phosphate on the reduction of vinylacetate*

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M-KH_2PO_4–K_2HPO_4, pH 8·0. Sodium vinylacetate (14 μmoles in Expt. 1; 10 μmoles in Expts. 2 and 3) and acetyl phosphate in the amounts indicated were added from the side arm after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°.

Acetyl phosphate added (μmoles)	H_2 uptakes					
	Expt. 1		Expt. 2		Expt. 3	
	Total (μl.)	Rate (μl./hr.)	Total (μl.)	Rate (μl./hr.)	Total (μl.)	Rate (μl./hr.)
0·2	190	121	—	—	—	—
0·1	211	133	110	122	124	128
0·05	214	134	—	—	100	128
0·025	—	—	—	—	27	14
0·02	—	—	115	134	—	—
0·013	—	—	—	—	120	124
0·006	—	—	—	—	109	116
0·004	—	—	11	8	—	—
0·003	—	—	—	—	117	124
0·0008	—	—	7	8	—	—
0·00016	—	—	10	8	—	—
0·00003	—	—	10	8	12	6
0	38	29	10	8	—	—

the cessation of the H_2 uptake and the addition of the extra vinylacetate. In a separate experiment, an attempt was made to determine the time interval required for destruction of the activating principle by making the second addition of vinylacetate at various times relative to the point at which the H_2 uptake due to the first addition of vinylacetate ceased. This interval proved too short to measure; extra vinylacetate was reduced only if it was added during the linear phase of the reaction.

The fact that H_2 uptake occurs at a linear rate until it is complete shows that the activating principle is quite stable in presence of vinylacetate. Other evidence supports this view. With the manometer containing the larger amount of vinylacetate, H_2 uptake was still proceeding at the initial rate after 90 min. (curve *A*, Fig. 1*a*) whereas, after the same time, the system with half that amount of vinylacetate required more acetyl phosphate in order to reduce extra vinylacetate (curve *H*, Fig. 1*b*). It may be concluded that acetyl phosphate, or some activating compound derived from it, is rapidly destroyed by the dried-cell suspension

intermediate rate was observed, except possibly in the vessel in which 0·025 μmole was added in Expt. 3, where the observed rate was only slightly higher than with the lowest amount of acetyl phosphate. This particular observation appears anomalous, since in the same experiment three lower amounts of acetyl phosphate were effective, but no explanation can be offered. The amount of acetyl phosphate required to stimulate reduction is variable; thus in Expt. 3 0·003 μmole was effective, whereas in Expt. 2 0·004 μmole did not stimulate reduction. The amount of acetyl phosphate usually added in subsequent experiments was 0·1 μmole; this amount never failed to stimulate reduction.

Activation by the products of the reaction between acetyl phosphate and dried cells. It appeared possible that the reducing system might be activated, not by acetyl phosphate as such, but rather by some product derived from it, especially since EBY, which also stimulates, is unlikely to contain any acetyl phosphate. An experiment was therefore carried out to determine whether the interaction of dried cells with acetyl phosphate would yield a product capable of activating the

reducing system. For this purpose a relatively large amount of acetyl phosphate was used, since this compound was known to be unstable in presence of dried cells, and the time of incubation was kept short. After incubation, the enzymes and excess of acetyl phosphate were destroyed by heating at pH 3·5, and the soluble products remaining after centrifuging tested for activating properties. Corresponding products from control incubations in which the acetyl phosphate or the dried cells were omitted were also tested.

Table 6. *Stimulation of vinylacetate reduction by the products obtained by incubating acetyl phosphate with dried cells of* Clostridium kluyveri

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M tris–KH_2PO_4, pH 8·0. Sodium vinylacetate (10 μmoles) and incubation products as indicated were added from the side arm after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°. The figures have been corrected by subtracting the values from corresponding flasks without vinylacetate. These latter values were all within the range 2–19 μl.

The incubation products were prepared as follows. Dried cells of *Cl. kluyveri* (100 mg.) and 10 μmoles of acetyl phosphate were incubated for 15 min. at 26° in a total vol. of 2·0 ml. containing 0·04 M tris–KH_2PO_4, pH 8·0, and 0·03% $Na_2S,9H_2O$. Blank mixtures from which dried cells and acetyl phosphate were omitted respectively were treated likewise. After incubation the mixtures were cooled, the pH was adjusted to 3·5 with HCl, the mixture heated at 100° for 15 min. and cooled, the pH adjusted to 8 with NaOH, the mixture centrifuged and 0·5 ml. of the resulting supernatant used for the manometric tests.

	H_2 uptake	
	Total vol. (μl.)	Rate (μl./hr.)
1. Product tested from incubation of		
Dried cells + acetyl phosphate	130	130
Dried cells alone	18	8
Acetyl phosphate alone	6	5
2. Acetyl phosphate tested directly	109	96

The results (Table 6) show that in the presence of dried cells acetyl phosphate is converted into a product which is heat-stable and capable of activating the reduction of vinylacetate. The rate of reduction is in fact rather higher than in the presence of acetyl phosphate.

Activation by acetyl-coenzyme A. Preparations of *Cl. kluyveri* are known to contain phosphotransacetylase, which catalyses the transfer of the acetyl group from acetyl phosphate to CoA (reaction 6), and the results of the experiments described above are compatible with the activation of the reducing system by acetyl-CoA. This possibility was tested more directly by using a purified sample of acetyl-CoA.

The compound was found to activate the system (Table 7), and the rate of reduction was again slightly higher than that observed with acetyl phosphate. The stimulation observed with EBY may be due to the presence of acetyl-CoA, since this compound has been isolated from yeast by Lynen & Reichert (1951).

Table 7. *Stimulation of vinylacetate reduction by acetyl-CoA*

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M tris–KH_2PO_4, pH 8·0. Sodium vinylacetate (5 μmoles), acetyl phosphate (0·1 μmole) and acetyl-CoA (0·08 μmole; preparation described under Methods) were added, as indicated, from the side arm, after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°.

	H_2 uptake	
System	Total vol. (μl.)	Rate (μl./hr.)
Vinylacetate present, no supplements	6	2
Vinylacetate present + acetyl phosphate	68	82
Vinylacetate present + acetyl-CoA	77	98
Vinylacetate absent + acetyl-CoA	8	2

Table 8. *Inhibition of vinylacetate reduction by acetate*

Manometers contained in a total vol. of 2·0 ml.: 50 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M tris–KH_2PO_4, pH 8·0. Sodium vinylacetate (5 μmoles), acetyl phosphate (0·1 μmole) and sodium acetate, potassium acetate or NaCl as indicated were added from the side arm after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°. The figures given have been corrected by subtracting the values obtained in the absence of vinylacetate which lay within the range 6–11 μl./hr.

Additions	Rate of H_2 uptake (μl./hr.)
None	85
Sodium acetate (0·005 M)	1
Potassium acetate (0·005 M)	0
NaCl (0·005 M)	87

Inhibition of the reduction of vinylacetate by acetate. It is apparent from the earlier experiments that, in the absence of vinylacetate, acetyl phosphate is removed from the system by some reaction in which the energy-rich acyl bond is presumably destroyed. One such reaction already known to be catalysed by preparations of *Cl. kluyveri* is the reduction of acetyl phosphate plus acetate to butyrate. Consequently it was expected that the addition of acetate to the system might accelerate

the disappearance of acetyl phosphate and in this way prevent the activation of the system reducing vinylacetate. The effect of acetate was therefore tested.

When 0·005 M sodium acetate was added to the usual test system, the hydrogen uptake was completely inhibited (Table 8; the H_2 uptake required to remove the small amount of acetyl phosphate present is negligible). This appears to be a true effect of the acetate ion, since complete inhibition was also found with potassium acetate, whereas there was no inhibition with NaCl.

Inhibition of the reduction of vinylacetate by arsenate. Previous work (Stadtman & Barker, 1949*c*, *e*) has shown that the oxidation of vinylacetate to acetyl phosphate plus acetate and the reduction of acetyl phosphate plus acetate to butyrate are both inhibited by arsenate, and it was therefore of interest to investigate the effect of this inhibitor on vinylacetate reduction.

Table 9. *Inhibition of vinylacetate reduction by arsenate*

Manometers contained in a total vol. of 2·0 ml.: 100 mg. of dried cells of *Cl. kluyveri*; 0·001 M BAL; 0·05 M tris–KH_2PO_4, pH 8·0; 0·5 ml. of extract of boiled *Cl. kluyveri* (1 g. of dried cells of *Cl. kluyveri*, lot A 0, were suspended in 5 ml. of water and heated at 100° for 20 min. The mixture was then centrifuged and the clear supernatant used). Potassium vinylacetate (10 μmoles), acetyl phosphate (0·1 μmole) and potassium arsenate were added as indicated from the side arm after saturation of the enzyme system with H_2. Gas phase H_2; temp. 26°.

System	Rate of H_2 uptake (μl./hr.)
Vinylacetate absent, no arsenate	21
Vinylacetate present, no arsenate	378
Vinylacetate absent + arsenate (0·025 M)	15
Vinylacetate present + arsenate (0·025 M)	21

The experiment was carried out after the dried cell preparation used in the previous experiments (Lot YE 9) had been stored at −18° for 12 months. The preparation had lost most of its activity during this period, in contrast to the earlier batches of cells prepared exactly according to Stadtman & Barker (1949*a*) and referred to in the Materials and Methods section above. However, by increasing the concentration of dried cells and adding a boiled extract of a different dried cell preparation (Lot AO, prepared according to Stadtman & Barker, 1949*a*) a rapid reduction of vinylacetate was obtained. With these modified conditions, it was found that 0·025 M arsenate completely inhibited the uptake of H_2 in the presence of vinylacetate (Table 9).

DISCUSSION

Kennedy & Barker (1951*a*) suggested that in *Cl. kluyveri* vinylacetate was reduced in the form of a complex with a coenzyme, possibly CoA, as indicated in Fig. 2. The results presented in this paper show quite clearly that acetyl phosphate or acetyl-CoA is necessary for the reduction of vinylacetate, indicating that the vinylacetate molecule must be activated in some way before reduction can occur. This evidence therefore supports the scheme proposed by Kennedy & Barker and suggests that the conversion of vinylacetate into the active complex requires a high-energy acyl bond.

It had previously been observed that acetyl phosphate was required for the oxidation of butyrate by dialysed extracts of *Cl. kluyveri* (Stadtman & Barker, 1949*b*), but in these experiments the amounts of acetyl phosphate used were large (10 μmoles). From the present experiments it is quite evident that the role of acetyl phosphate in the reduction of vinylacetate is essentially catalytic, since only a fraction of a micromole is necessary to permit the reduction of several micromoles of vinylacetate. It follows that the energy of the acyl link used to activate vinylacetate must be conserved in some way.

It is now known that in animal tissues the fatty acids are oxidized in the form of their thioester derivatives of CoA (Mahler, 1953; Lynen, 1953), and extracts of *Cl. kluyveri* have been shown to oxidize butyryl-CoA (Stadtman, 1953*a*). The current views on the synthesis and oxidation of butyrate by bacterial systems are summarized in Fig. 2 (cf. Stadtman, 1953*a*).

The activation and reduction of vinylacetate can therefore be readily explained by the following series of reactions, in which coenzyme A is represented by CoASH for convenience.

Activation process

$$CH_3.CO_2PO_3H_2 + CoASH \rightleftharpoons CH_3.CO.SCoA + H_3PO_4, \quad (6)$$

$$CH_3.CO.SCoA + CH_2{:}CH.CH_2.CO_2H \rightleftharpoons CH_2{:}CH.CH_2.CO.SCoA + CH_3.CO_2H. \quad (7)$$

Cyclic reduction process

$$CH_2{:}CH.CH_2.CO.SCoA + H_2 \rightarrow CH_3.CH_2.CH_2.CO.SCoA, \quad (8)$$

$$CH_3.CH_2.CH_2.CO.SCoA + CH_2{:}CH.CH_2.CO_2H \rightleftharpoons CH_2{:}CH.CH_2.CO.SCoA + CH_3.CH_2.CH_2.CO_2H. \quad (9)$$

Reaction 6 is the phosphotransacetylase reaction and reaction 7 is catalysed by the CoA transphorase of *Cl. kluyveri*. Both these enzymes have been

partially purified and examined in some detail by Stadtman (1952*b*, 1953*b*). Thus the initial activation of vinylacetate by conversion into vinylacetyl-CoA is explicable in terms of enzyme systems known to occur in *Cl. kluyveri*.

There has been no direct demonstration of reaction 8 in this organism, though Stadtman & Korkes (quoted by Stadtman, 1953*a*) have shown that butyryl-CoA is oxidized to acetoacetyl-CoA, presumably via a butenoyl derivative of CoA. From animal tissues, Mahler and co-workers (Green, Mii, Mahler & Bock, 1954; Mahler, 1954) have obtained, in a highly purified state, a cupro-flavoprotein which catalyses the oxidation of butyryl-CoA to a butenoyl-CoA. The position of the double bond in the butenoyl derivative has not been finally settled, but it is known that crotonyl-CoA can act as substrate for the reverse reaction [Mahler, 1954; also cf. the work of Seubert & Lynen (1953) with the *N*-acetylthioethanolamine analogues of the thioesters of CoA]. It is possible that in presence of the appropriate hydrogen donor this enzyme might catalyse the reduction of vinylacetyl-CoA to butyryl-CoA directly, or alternatively reduction may be preceded by conversion of vinylacetyl-CoA into crotonyl-CoA. This latter conversion would appear possible by way of 3-hydroxybutyryl-CoA as intermediate, since the unsaturated fatty acyl-CoA hydrase of animal tissues reversibly hydrates both these unsaturated derivatives (Wakil & Mahler, 1954).

Reaction 9 may be catalysed directly by CoA transphorase, though this reaction has not so far been reported. Alternatively, this transfer of CoA might be effected in two stages in the presence of catalytic amounts of acetate by reaction 10 followed by reaction 7.

$$CH_3.CH_2.CH_2.CO.SCoA + CH_3.CO_2H \rightleftharpoons CH_3.CO.SCoA + CH_3.CH_2.CH_2.CO_2H. \quad (10)$$

Reaction 10 is known to be catalysed by the CoA transphorase of *Cl. kluyveri* (Barker, Stadtman & Kornberg, 1955).

Reactions 6–9 could thus be accomplished by known enzyme systems, and such a sequence would explain the dependence of vinylacetate reduction on activation by acetyl phosphate or acetyl-CoA. The requirement for only catalytic amounts of the activator is also explicable in terms of these reactions, since the thioester linkage is preserved in reactions 8 and 9, and the system becomes self-sustaining once a catalytic amount of vinylacetyl-CoA has been formed.

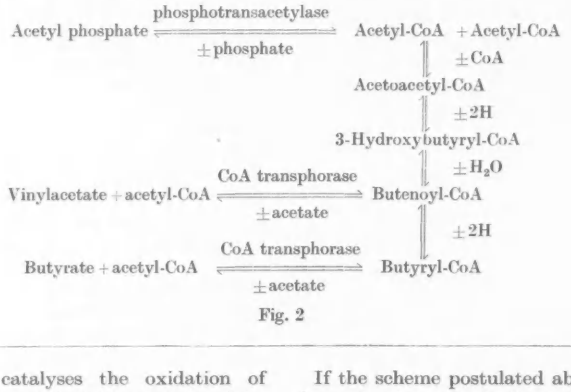

Fig. 2

If the scheme postulated above is correct, then the reduction of vinylacetate should be CoA-dependent. Experiments designed to test this have so far proved inconclusive. Preparations from which CoA was removed by treatment with Dowex 1 resin (Dow Chemical Co., Midland, Mich.), as described by Chantrenne & Lipmann (1950), were not reactivated by CoA alone, although boiled extracts of *Cl. kluyveri* would restore activity (Peel & Barker, 1953). Cofactors other than CoA, which may well be required for the hydrogen-activating or electron-transporting systems, would be removed by the resin treatment.

The exact structure of the butenoyl-CoA intermediate in butyrate synthesis and oxidation remains undecided (see Anfinsen & Kielley, 1954, for a full discussion), though arguments based on specificity and energy relationships indicate that crotonyl-CoA is more likely to be the true intermediate, at least in animal tissues (Green *et al.* 1954). Vinylacetyl-CoA was formerly considered the more likely intermediate in the *Cl. kluyveri* system, since preparations of the organism will metabolize vinylacetate but not crotonate. Stadtman (1953*a*), however, has pointed out that this may simply be due to the inability of the system to

activate crotonate, since the CoA transphorase of *Cl. kluyveri* catalyses the transfer of CoA from acetyl-CoA to vinylacetate but not to crotonate. The ultimate solution of this problem must await further studies on the isolated enzyme systems.

The instability of acetyl phosphate in the absence of vinylacetate has been explained in terms of its conversion into butyrate. The inhibitory action of acetate now requires further consideration in terms of the mechanism proposed for the reduction of vinylacetate. Acetate is a CoA acceptor and would tend to remove vinylacetyl-CoA and butyryl-CoA by reversing the vinylacetate-activating reaction 7 and promoting reaction 10: thus in the presence of acetate there would be a leakage of the thioesters concerned in the cyclic reduction process (reactions 8 and 9). The resulting acetyl-CoA would be converted into acetoacetyl-CoA by β-ketothiolase and then reduced by hydrogen. The condensation involves a net loss of acyl thioester bonds, and would result in the complete removal of such bonds from the system, provided that sufficient free acetate were present.

The inhibition by arsenate can be explained along similar lines. In the presence of arsenate, phosphotransacetylase would rapidly break down acetyl-CoA to CoA and free acetate. This acetate could then react with other CoA derivatives in the presence of CoA transphorase, yielding more acetyl-CoA, which in turn would undergo arsenolysis. The overall result would again be the removal of the acyl-CoA derivatives on which the reduction of vinylacetate depends.

The explanations offered above for the phenomena connected with the reduction of vinylacetate are over-simplified. Only one reaction bringing about a removal of acetyl phosphate has been taken into account, namely the reduction of acetyl phosphate plus acetate to butyrate (reaction 1), whereas preparations of *Cl. kluyveri* also catalyse a slow hydrolysis of acetyl phosphate (Stadtman & Barker, 1950; Lieberman, 1954). It is also possible that, after the initial activation of vinylacetate, acetyl phosphate may be generated by dismutation according to reaction 5. Both these reactions would affect the number of high-energy acyl bonds present in the system and thus might exert a controlling effect on the activation and reduction of vinylacetate. The fact that all the added vinylacetate is not reduced to butyrate would be understandable if, in the later stages of the reaction, the production of acyl bonds by the dismutation of vinylacetate could not keep pace with the hydrolysis of acetyl phosphate. Such considerations, however, involve modifications to the proposed scheme for the reduction of vinylacetate rather than any change of the basic principles involved.

So far, no mention has been made of the hydrogen-activating system of *Cl. kluyveri* which is involved in the test system used for the experiments described in this paper. Arsenate has no effect on this system, since it does not inhibit the reduction of acetoacetate to 3-hydroxybutyrate by molecular hydrogen (Stadtman & Barker, 1949*d*). The possibility that the stimulation of vinylacetate reduction by acetyl phosphate might be due to an effect on the hydrogen-activating system would appear to be ruled out by the following observations. Preparations of *Cl. kluyveri* possess a strong yellow colour which is largely due to the presence of flavin compounds, principally flavin-adenine dinucleotide (Peel, 1955). In the presence of H_2 this yellow colour disappears, presumably owing to the reduction of these flavins, and, with the preparation YE9 used in the above experiments, this occurs without the addition of acetyl phosphate. It is known that the butyryl-CoA dehydrogenase of animal tissues is a cuproflavoprotein (Mahler, 1954), and a survey of the flavin content of a variety of micro-organisms has revealed a correlation between a high flavin content and the metabolism of fatty acids (Peel, 1955). Consequently, it appears probable that a similar enzyme operates in *Cl. kluyveri*. If so, acetyl phosphate is not required for its reduction by molecular hydrogen.

SUMMARY

1. In presence of molecular hydrogen, dried cells of *Cl. kluyveri* reduce vinylacetate (3:4-butenoate) to butyrate. With some dried cell preparations extract of boiled yeast is required for this reaction.

2. The extract of boiled yeast can be replaced by catalytic amounts of acetyl phosphate or acetyl-CoA, but not by various known cofactors, including CoA.

3. In the absence of vinylacetate and in the presence of hydrogen, acetyl phosphate is destroyed by the preparations and stimulates reduction only if added at the same time as, or after addition of, the vinylacetate.

4. Whenever reduction occurred, the rates of hydrogen uptake and the total hydrogen uptakes were the same, whatever the amount of acetyl phosphate added. No intermediate rate was observed. The minimum amount of acetyl phosphate required to stimulate reduction could not be established with certainty. The lowest effective amount (in 2 ml.) was 0·003 μmole.

5. The reduction of vinylacetate is completely inhibited by 0·005 M acetate or 0·025 M arsenate.

6. These observations can be explained on the assumption that vinylacetate is reduced, not in the free state, but in the form of vinylacetyl-CoA, which is produced by a transfer of CoA from acetyl- or butyryl-CoA.

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The Metabolism of Progesterone by Animal Tissues *in vitro*

3. INVESTIGATION OF THE PRODUCTS OF METABOLISM AFTER INCUBATION OF 5β-PREGNANE-3α:20α-DIOL WITH RABBIT-LIVER HOMOGENATE*

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It is well established that when progesterone (pregn-4-ene-3:17-dione) is administered to human subjects a considerable proportion remains unaccounted for by the known progesterone metabolites, mainly 5β-pregnane-3α:20α-diol ('pregnanediol') subsequently recoverable from the urine. Moreover, Sommerville & Marrian (1950) found that when 'pregnanediol' itself was administered orally to human subjects the proportion of administered steroid subsequently excreted in the urine as 'pregnanediol' was no higher than when progesterone was given to the same subjects by the same route. It was suggested by these authors that these low recoveries of urinary 'pregnanediol' might be due to excretion of that steroid by other routes or to conversion into further metabolites as yet unidentified, or to a combination of both processes.

To explore the possibility that further metabolism of 'pregnanediol' occurs in the body, Grant & Marrian (1950) and Grant (1952) investigated the metabolism of 'pregnanediol' dihemisuccinate by rabbit- and rat-liver preparations. Liver slices, acetone powders and homogenates were able to metabolize 'pregnanediol' under aerobic conditions, and addition of diphosphopyridine nucleotide

* Part 2: Taylor (1955).

(DPN) to rat-liver homogenates increased their ability to metabolize 'pregnanediol'. 50% of the 'pregnanediol' incubated with rat-liver homogenate was metabolized and of this amount only 10% was accounted for by the isolation of 3α-hydroxy-5β-pregnan-20-one ('pregnanolone') (Grant, 1952). The fate of the main part of the 'pregnanediol' which disappeared was not known, but it is possible that it can be attributed largely to losses incurred during the extraction and purification processes employed.

It has not been established whether or not 'pregnanediol' is a metabolite of progesterone in the rat; indirect evidence indicates that it is not. Thus Riegel, Hartop & Kittinger (1950) showed that after injection of [21-^{14}C]progesterone into rats, most of the radioactivity was found in the faeces in a ketonic, non-alcoholic steroid which was not progesterone. Furthermore, Taylor (1954) was able to isolate only 5α-pregnane-3:20-dione and 3α-hydroxy-5α-pregnane-20-one after incubation of progesterone with rat liver. It seemed possible, therefore, that Grant (1952) failed to isolate metabolites other than 'pregnanolone' after incubation of 'pregnanediol' with rat liver because in that tissue the enzyme systems responsible for the further metabolism of 'pregnanediol' (except at C-20) are either absent or are specific for steroids possessing the 5α-configuration.

It was therefore decided to investigate the metabolism of 'pregnanediol' by the liver of the rabbit, since in this animal, as in humans, 'pregnanediol' is a major metabolite of progesterone, and when progesterone is incubated with rabbit liver under aerobic conditions 'pregnanediol' is a major metabolite (Taylor, 1955).

'Pregnanediol' dihemisuccinate has been incubated in air with a suspension of disintegrated rabbit liver under conditions similar to those employed for rat liver by Grant (1952). Only two metabolites, 3α-hydroxy-5β-pregnan-20-one and 5β-pregnane-3:20-dione, have been isolated; these two metabolites along with the unchanged 'pregnanediol' isolated accounted for 54% of the steroid added to the incubation mixture.

EXPERIMENTAL

Materials and methods

Pregnanediol dihemisuccinate (PDHS) (m.p. 147–148·5°) was prepared as described by Grant & Marrian (1950). Authentic 5β-pregnane-3:20-dione (m.p. 119–120·5°) was prepared by oxidation of 'pregnanediol' with CrO_3 in acetic acid solution (Marker, Kamm & McGrew, 1937). Other reference steroids were the specimens previously described (Taylor, 1955).

For adsorption chromatography, alumina (100/150 mesh, Savory and Moore Ltd., London) was inactivated by exposing it to an atmosphere saturated with water vapour at room temperature for 10–14 days. Chromatography was carried out in 2·0 cm. diam. columns containing 30 g. of alumina. The material to be chromatographed was dissolved in the minimum volume of benzene and the column eluted by the gradient-elution technique with ethanol–benzene mixtures ranging from 0 to 2·2% (v/v) of ethanol by a linear gradient over 200 ml., and then finally with 100 ml. of 5% (v/v) ethanol–benzene. Volumes of 10 ml. were collected. Solvents were not dried but were twice distilled in all-glass apparatus shortly before use. Substances isolated were identified by comparison of the following properties with those of authentic steroids: m.p. and mixed m.p., absorption spectra in conc. H_2SO_4 over the range 222–520 mμ. and infrared spectra in CS_2 solution over the 'fingerprint' region of the free steroids or, for hydroxysteroids, their acetates. All melting points are corrected and were determined in a Kofler-type apparatus.

Other materials and methods were as described in previous publications (Taylor, 1954, 1955), unless otherwise stated.

Incubation of PDHS with suspension of disintegrated rabbit liver

The experiment was conducted in four parts, a total of 215 mg. of 'pregnanediol' as PDHS being incubated with 268 g. (wet wt.) of rabbit liver. The following is a description of a typical experiment. PDHS (81·5 mg. containing 50 mg. of 'pregnanediol') was dissolved in 100 ml. of 'phosphate-saline' and 20 ml. of this solution was added to each of five 100 ml. conical flasks. The rabbit liver (61·5 g.) was homogenized for 2 min. at high speed in the Atomix blender (Measuring and Scientific Equipment Ltd., London) with 40 ml. of 0·15M-KCl containing sufficient nicotinamide to provide a concentration of 0·08M in the final reaction mixture. The resulting 'suspension' was filtered through two layers of gauze, made up to 100 ml. with 0·15M-KCl and added in 20 ml. portions to the reaction vessels. The flasks were not stoppered but were shaken vigorously during the incubation for 2 hr. at 37°. A neutral lipid extract was then prepared (Taylor, 1954), and the combined extracts from the four parts were separated into ketonic and non-ketonic, α- and β-fractions by treatment with Girard Reagent T and digitonin as previously described (Taylor, 1955). (Because of the scarcity of 'pregnanediol' it was not possible to carry out a parallel 'control' experiment; the effect of this on the validity of the results is considered in the Discussion section.) The weights of the various fractions thus obtained are given in Table 1.

Table 1. *Weights (mg.) of fractions obtained after treatment of lipid extract with Girard reagent T and digitonin*

Total neutral lipids	426
Ketonic fraction	149
α-Ketonic	116
β-Ketonic	0
Non-ketonic fraction	217
α-Non-ketonic	165
β-Non-ketonic	21

Investigation of fractions

α-*Ketonic*. This fraction, a dark-brown gum, was separated into three main components by chromatography. Component I (38 mg.) (fractions 1–5) was a dark-brown oil which could not be resolved further either by rechromatography or by acetylation and rechromatography. Component II (7 mg.) (fractions 6–9), after two crystallizations from hexane, yielded 5·3 mg. of thick rods, m.p. 118–120°, identified as 5β-pregnane-3:20-dione. Component III (60·4 mg.) (fractions 10–15), on crystallization from hexane, gave 49 mg. of fine needles, m.p. 146–148°, identified as 3α-hydroxy-5β-pregnan-20-one.

α-*Non-ketonic*. This fraction was white and semicrystalline. On chromatography only one main fraction (84 mg.) eluted at ethanol concns. from 1·5 to 2·2% (v/v) was obtained. One crystallization from aqueous ethanol and one from benzene yielded 62 mg. of crystals, m.p. 234·5–237°, which were proved to be 'pregnanediol'.

β-*Non-ketonic*. A quantitative Liebermann–Burchard reaction on this semicrystalline material indicated that it consisted very largely of cholesterol. Direct crystallization from ethanol yielded 17 mg. of fine needles, m.p. 145–148°, which were identified as cholesterol.

Table 2. *Steroids isolated after incubation of* 215 *mg. of 'pregnanediol' with rabbit liver*

Steroid isolated	Wt. (mg.)	As percentage of added 'pregnanediol'
5β-Pregnane-3:20-dione	5·3	2·5
3α-Hydroxy-5β-pregnan-20-one	49	22
'Pregnanediol'	62	29
Totals	116·3	53·5

The results of the experiment are summarized in Table 2. The total recovery of steroids (54%) compares favourably with that obtained in similar experiments in which progesterone was incubated with rat and rabbit liver (Taylor, 1954, 1955) (see Discussion).

DISCUSSION

It has been shown that on incubation with suspensions of disintegrated rabbit liver 'pregnanediol' is metabolized to 5β-pregnane-3:20-dione ('pregnanedione') and 3α-hydroxy-5β-pregnan-20-one ('pregnanolone'). In a similar experiment with rat liver Grant (1952) was able to isolate only 'pregnanolone'. His failure to isolate 'pregnanedione' may have been due to the fact that only a very small amount of this substance was formed. (In the present experiment only 2·5% of the incubated 'pregnanediol' was recovered as the dione.) It is more probable, however, that the oxidation of the 3-hydroxyl group of 5β-pregnane compounds does not occur in rat liver, since in this tissue reduction of progesterone yields only 5α-pregnane derivatives (Taylor, 1954). Rabbit liver, on the other hand, metabolizes progesterone to steroids which are mainly of the 5β-series (Taylor, 1955).

The amount of 'pregnanedione' isolated in the present experiment was small compared with the amount of 'pregnanolone' isolated. This may explain the failure of Taylor (1955) to isolate 'pregnanedione' after incubation of progesterone with rabbit liver.

Since no 20α-hydroxy-5β-pregnan-3-one was isolated in the present investigation, it seems probable that oxidation of the C-20 hydroxyl group must precede oxidation of the C-3 hydroxyl group.

It was not possible to carry out a parallel 'control' experiment, but from the author's previous experience it is considered that the 54% recovery of added steroid precludes the possibility that any other major metabolites were formed. Thus in 'control' experiments in which progesterone was added to incubated rat liver (Taylor, 1954) and rabbit liver (Taylor, 1955) 56 and 52% respectively of the steroid was recovered as crystalline material. It appears, therefore, that under the conditions employed rabbit liver does not convert 'pregnanediol' into substances other than those isolated.

It is recognized that this type of *in vitro* experiment may bear little or no relationship to the metabolism of steroids in the intact animal; nevertheless, it is possible that a large proportion of administered progesterone or 'pregnanediol' unaccounted for as urinary 'pregnanediol' (Sommerville & Marrian, 1950) is excreted in the urine or in the faeces (via the bile) (cf. Marrian, 1954) as 'pregnanolone' and 'pregnanedione'. The determination of these substances in urine and bile or faeces would therefore be of value in attempts to assess disorders of progesterone production and metabolism.

SUMMARY

1. In a large-scale experiment in which 5β-pregnane-3α:20α-diol ('pregnanediol') was incubated with a suspension of disintegrated rabbit liver with added nicotinamide, 54% of the added steroid has been accounted for by the isolation of 5β-pregnane-3:20-dione (2·5%) and 3α-hydroxy-5β-pregnan-20-one (22%) and unchanged 'pregnanediol' (29%).

2. The significance of this finding has been discussed.

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The Oxidation of L-Amino Acids by *Mytilus edulis*

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It is now well established that amino acid oxidases are widely distributed in molluscs. The cephalopod liver contains not only a general D-amino acid oxidase (Blaschko & Hawkins, 1951), but probably also a second oxidase that acts upon D-aspartic and D-glutamic acids (Blaschko & Himms, 1955); D-amino acid oxidase also occurs in the digestive gland of *Helix*.

When other molluscan species were examined, it was found that the digestive gland of *Mytilus edulis* also had amino acid-oxidase activity: DL-methionine, DL-α-aminobutyric acid, DL-norvaline and DL-norleucine were found to be oxidized (Blaschko & Hawkins, 1952). In the same year, Roche, Thoai & Glahn (1952) described an enzyme in the digestive gland of *Mytilus* that oxidized L-arginine and a few other amino acids. Since in the earlier experiments with *Mytilus* (Blaschko & Hawkins, 1952) all the compounds tested were racemic mixtures, it seemed desirable to find out whether the *Mytilus* oxidase had acted upon the D- or the L-forms of the racemic amino acids studied.

In the experiments described below, no evidence for the occurrence of a D-amino acid oxidase in *Mytilus* was obtained. On the other hand, many L-amino acids were oxidized. The substrate specificity of the *Mytilus* enzyme shows characteristic differences from other L-amino acid oxidases that are described in the literature.

MATERIAL AND METHODS

Some of the preliminary experiments were carried out at Plymouth, where freshly caught animals were used. Most of the work was done in Oxford, on specimens sent from Plymouth. The animals were dissected soon after arrival; only animals that appeared to be vigorous were used.

In *Mytilus*, the digestive gland contains parts of the intestine which cannot be separated by dissection. The dissected tissue was weighed, frozen in a mortar, cooled to −10° and then thoroughly ground at room temperature. To each gram of fresh tissue 2 ml. of 0·067 M-sodium phosphate buffer at pH 7·15 was added to give a crude suspension.

In the experiments on substrate specificity, the crude suspension was centrifuged at 3000 *g* for 1 hr. at 0°. The sediment was resuspended in the original volume of phosphate buffer and recentrifuged. The sediment was again suspended in the phosphate buffer and dialysed against the same buffer for 24 hr. at 3°. This procedure gave preparations with a satisfactory enzymic activity and a relatively low oxygen consumption in the 'enzyme blank'.

For the manometric determination of enzymic activity 1 ml. of the *Mytilus* preparation was used in the main compartment of a conical flask, together with 0·6 ml. of the phosphate buffer. The inner tube contained a filter paper and 0·3 ml. of N-KOH, the side bulb 0·4 ml. of either water ('enzyme blank') or of a solution containing 10 μmoles of the L-amino acid to be tested, so as to make the initial substrate concentration 0·005 M. Some of the amino acids were not sufficiently soluble; these were prepared as a suspension of the solid material finely ground in a mortar. This was necessary with cystine (L-, *meso*- and DL-cystine), with DL-homocystine, with DL-α-aminocaprylic acid and with DL-tyrosine. When DL-amino acids were used each flask contained 20 μmoles of the acid.

Unless otherwise stated, the material was obtained from commercial sources. We are indebted to Dr Elizabeth Work for the samples of αα′-diaminopimelic acid and to Professor H. A. Krebs for a number of amino acids prepared by Elks, Hems & Ryman (1948); Dr E. P. Abraham gave us a sample of DL-α-aminoadipic acid prepared by Gaudry (1949).

In the preparation of L-canavanine from Jack beans we followed the advice of Dr E. E. Snell and used in the purification procedure a column of Dowex 50 (×8, 200–400 mesh) in its H form. Crystalline L-canavanine acid sulphate was readily obtained upon seeding with a few crystals given by Dr H. Kihara. Another sample of L-canavanine sulphate was kindly given by Dr E. A. Bell and Professor W. R. Fearon. The total yield of analytically pure L-canavanine acid sulphate from 1 kg. of whole Jack beans was 10·3 g.

In the manometric experiments, the gas phase was O_2 and the temperature 25°. The time course of the oxidation was followed for at least 0·5 hr., but often for much longer, until the enzymic reaction had gone to completion. In most experiments, one flask contained L-arginine as substrate and the initial rate of oxidation of the other amino acids was expressed as a percentage of that of L-arginine. During the initial period, the rate of oxidation was constant.

RESULTS

Properties of the oxidase

In the preliminary studies a crude preparation of the gland was used, in which the tissue had been ground in a mortar with sand. It was soon noted that the enzyme was associated with insoluble material which could easily be spun down on the centrifuge. This was demonstrated in an experiment with a homogenate of the gland tissue in 0·32-molal sucrose, prepared in a glass homogenizer; 5 g. of fresh tissue were used, and the final vol. of the homogenate was 24 ml. This homogenate was centrifuged at 0° and 950 *g* for 20 min. The sediment was resuspended in sucrose so that the final volume was that of the original homogenate. When 1·0 ml. of both supernatant fluid and resuspended sediment were tested with 0·01 M L-arginine at pH 7·15, the amounts of oxygen (μl.) consumed in 1 hr. were:

	Without arginine	With arginine	Diff.
Supernatant	32	41	9
Resuspended sediment	16	97	81

In other words, nine-tenths of the enzymic activity was found in the sediment and one-tenth in the supernatant.

An attempt was made to obtain a soluble preparation of enzyme by using a supersonic disintegrator, but even after treatment for 30 min. with vibrations of 25 kc./sec. generated by a 600 w Mullard supersonic generator, the supernatant fluid after high-speed centrifuging was without enzymic activity; most of the activity was recovered in the sediment. It follows that the enzyme is present in a structural element which is readily sedimented and not easily disintegrated.

Substrates of the oxidase

The relative rates of oxidation of the different amino acids tested, in comparison with L-arginine, are shown in Table 1.

Aliphatic monobasic monocarboxylic acids. A number of straight-chain amino acids were found to be oxidized. With L-alanine, the rate of oxygen consumption was very low, but there was an increase with α-amino-*n*-butyric acid, α-aminovaleric acid (norvaline) and α-aminocaproic acid (norleucine, 2-aminohexanoic acid); with norleucine the opti-

Table 1. *Rate of oxidation of amino acids by the* L-*amino acid oxidase of the digestive gland of* Mytilus edulis

The rate of oxidation is expressed in terms of percentage of the rate of oxidation of L-arginine. In these experiments the mean oxygen uptake in 12 min. of the 'enzyme blank' was 3·7 μl. (1·5–6·5) and the total oxygen uptake in the presence of L-arginine was 80·8 μl. (57–98) in the same period.

Substrate	Rate of oxidation
Aliphatic monocarboxylic monoamino acids	
DL-Alanine	4
DL-α-Aminobutyric acid	68
DL-Norvaline	86
DL-Norleucine	114
DL-α-Aminocaprylic acid	35
L-Valine	0
L-Isoleucine	0
L-Leucine	73
DL-Serine	0
DL-Threonine	0
Monocarboxylic diamino acids and other basic amino acids	
DL-αγ-Diaminobutyric acid	0
L-Ornithine	123
L-Lysine	125
DL-Hydroxylysine (synthetic)	28
L-Citrulline	114
L-Arginine	100
L-Canavanine	100
L-Histidine	80
Aromatic and heterocyclic amino acids	
L-Tryptophan	60
DL-5-Hydroxytryptophan	19
L-Proline	0
L-Phenylalanine	108
L-Tyrosine	110
Dicarboxylic diamino acids	
meso-αα′-Diaminopimelic acid	21
LL-αα′-Diaminopimelic acid	21
Sulphur-containing amino acids	
L-Cystine	38
meso-Cystine	40
DL-Homocystine	31
L-Cysteic acid	0
DL-Homocysteic acid	0
L-Cysteinesulphinic acid	0
L-Methionine	151
DL-Ethionine	97
LL-Djenkolic acid	40
Cystathionine (synthetic)	63
Dicarboxylic monoamino acids and amides	
L-Aspartic acid	0
L-Glutamic acid	0
DL-α-Aminoadipic acid	18
DL-α-Aminopimelic acid	19
DL-1-Aminoundecane-1:11-dicarboxylic acid	20
L-Asparagine	49
L-Glutamine	29

mum chain length seems to have been reached, and α-aminocaprylic acid (2-amino-octanoic acid) was more slowly oxidized. This is similar to the findings of Bender & Krebs (1950) on the cobra-venom oxidase; this enzyme also oxidizes norleucine at an optimal rate.

The results with α-aminobutyric acid, norvaline and norleucine confirm earlier observations from these Laboratories (Blaschko & Hawkins, 1952).

Of the amino acids with a branched chain, L-leucine was readily oxidized, but neither L-valine nor L-isoleucine was oxidized. This is reminiscent of the specificity pattern of the cobra-venom enzyme (Bender & Krebs, 1950). The cobra venom, as well as the *Mytilus* enzyme, appears not to act upon amino acids in which the two hydrogen atoms in the β-position are substituted.

Monocarboxylic diamino acids and other basic amino acids. Of the straight-chain aliphatic series, DL-αγ-diaminobutyric acid was not attacked, but the observation of Roche *et al.* (1952) on the oxidation of L-ornithine was confirmed; L-lysine was also readily oxidized.

L-Arginine is the substrate chiefly studied by Roche *et al.* (1952). Of a number of related compounds, L-canavanine was found to be oxidized as rapidly as arginine. The oxidation of L-canavanine has been studied in greater detail.

For the study of the oxidation product derived from L-canavanine, a dialysed washed suspension of *Mytilus* digestive gland was used, which had been prepared in distilled water instead of the phosphate buffer used in all other experiments.

Two experiments were carried out with this preparation. In the first, a manometric experiment with 0·005M L-canavanine as substrate, the reaction came to a standstill in 30 min. The supernatant from this incubation was used for a chromatographic analysis. This showed that all the canavanine had disappeared and that a new compound had been formed which gave an orange-brown colour with the sodium pentacyanoaminoferrate reagent for guanidinoxy compounds described by Fearon & Bell (1955); canavanine gives a magenta colour with this reagent. For the descending chromatography, phenol saturated with water was used in an atmosphere saturated with NH_3. At $24 \pm 1°$ the R_F of the reaction product, presumably α-oxo-δ-guanidinoxybutyric acid, was 0·83, and that of canavanine 0·68.

A parallel experiment was set up with the same enzyme preparation for the isolation of a reaction product as its 2:4-dinitrophenylhydrazone. An extract from 9 g. of fresh tissue was incubated at 25° for 45 min. with 68·5 mg. of L-canavanine acid sulphate, neutralized with NaOH. The total volume of the reaction mixture was 50 ml.; a 250 ml. Büchner flask was used which was agitated in the manometer bath while oxygen was passed through, just above the surface of the liquid. The reaction mixture was centrifuged at 3000 *g* for 30 min. at 0° and the supernatant was filtered through a filter paper. A portion (10 ml.) of 1% 2:4-dinitrophenylhydrazine in 2N-HCl was added. The mixture was left in the refrigerator at 2° overnight. On the next day an orange-coloured precipitate had separated. This was washed with water and dried. The crude *α-oxo-δ-guanidinoxybutyric acid* 2:4-*dinitrophenylhydrazone* had m.p. 212°. (Found: C, 37·6; H, 4·1. $C_{11}H_{13}O_7N_7$ requires C, 37·2; H, 3·7%.) Attempts to recrystallize the product brought about some decomposition, as shown by a lowered melting point.

Heterocyclic compounds. The oxidation of L-tryptophan by the *Mytilus* enzyme is in agreement with findings by Roche *et al.* (1952); we have already reported that DL-5-hydroxytryptophan is also readily oxidized (Blaschko & Hope, 1955). The oxidation of both tryptophan and 5-hydroxytryptophan leads to the formation of a brown pigment; this is reminiscent of the pigment formation which occurs when tryptamine or 5-hydroxytryptamine is oxidized by amine oxidase (Pugh & Quastel, 1937; Blaschko & Hellmann, 1953).

Dicarboxylic diamino acid. Apart from the sulphur-containing amino acids, which are discussed below, αα′-diaminopimelic acid was examined. Three forms of the acid were tested: the unresolved synthetic compound, the LL-form and the *meso*-form. All three were readily oxidized; however, the initial rate of oxidation was not maintained, so that an accurate measurement of the total amounts of oxygen consumed could not be made.

Sulphur-containing amino acids. Of the substrates examined by Blaschko & Hawkins (1952), methionine was the only one of which the D- and the L-forms were available. L-Methionine was very rapidly oxidized. With the D-form, there was a small oxygen uptake, which soon came to a standstill. That this oxygen uptake was due to a contamination of the sample with a small amount of the L-form is supported by the observation that a similar, small uptake of oxygen occurred when the sample was incubated with cobra venom.

L-Cysteic acid was not oxidized by the *Mytilus* preparation.

All the dicarboxylic amino acids of this group that were tested were found to be substrates; these were cystine (LL- and *meso*-form), homocystine, djenkolic acid and cystathionine.

Dicarboxylic monoamino acids and their amides. Neither L-aspartic acid nor L-glutamic acid was a substrate, but their amides were found to be oxidized.

The three long-chain dicarboxylic amino acids, DL-α-aminoadipic (C_6), DL-α-aminopimelic (C_7) and DL-1-aminoundecane-1:11-dicarboxylic (C_{13}) acids, were readily oxidized, all at approximately the same rate.

Total oxygen uptake

The total amount of oxygen taken up was not determined in every experiment, but with many of the substrates tested the reaction was followed to completion. With L-arginine and with many other substrates, the total amount of oxygen taken up was close to the theoretical, i.e. one-half molecule of O_2 for one molecule of L-amino acid added. Tryptophan consistently gave oxygen uptakes higher than the theoretical amount.

Other species

No significant increase over the blank oxygen consumption was observed when preparations of the digestive glands of *Pecten maximus* and of *Mya arenaria* were incubated with L-arginine, L-methionine, L-phenylalanine, L-leucine and L-valine. With a preparation from the digestive gland of *Cyprina islandica* the amounts of oxygen taken up during the first 30 min. of incubation were: without substrate, 5·5 μl.; with 0·01 M L-arginine, 14·5 μl.; with 0·01 M L-leucine, 16 μl.; with 0·01 M L-methionine, 28 μl. It seems therefore that *Cyprina islandica* contains an enzyme similar to that of *Mytilus*, but the preparation is less active.

The presence of a D-amino acid oxidase in the digestive gland of *Helix aspersa* (Blaschko & Hawkins, 1952) was confirmed. An experiment was performed with a preparation of this gland, prepared as described for *Mytilus*. The incubation was carried out at 37·5°, and each manometric flask contained the equivalent of 533 mg. of fresh tissue. The oxygen consumed in the first 10 min. was: without substrate, 15 μl.; with 0·01 M L-leucine, 17·5 μl.; with 0·01 M D-leucine, 104 μl.; with 0·01 M L-methionine, 17 μl.; with 0·01 M D-methionine, 112 μl.

DISCUSSION

The experiments reported in this paper show that the specificity of the *Mytilus* enzyme differs from that of the cephalopod enzyme and that of *Helix aspersa*: the *Mytilus* enzyme is a L-amino acid oxidase. Methionine was the only one of the four substrates tested by Blaschko & Hawkins (1952), of which both the D- and the L-forms were available. Only the L-form was oxidized at a significant rate. It seems likely that the enzyme responsible for the oxidation is identical with the L-arginine oxidase described by Roche *et al.* (1952), but this has not been fully investigated.

The study of the substrate specificity of the *Mytilus* enzyme has shown a number of analogies to that of other known oxidases of this group, but there are also differences. Many basic amino acids were oxidized, among them L-lysine and L-canavanine. Of the basic amino acids tested, only αγ-diaminobutyric acid was not oxidized.

The relative rates of oxidation of the straight-chain monoamino acids are reminiscent of the specificity pattern of the cobra-venom enzyme; this enzyme also has an optimum for α-aminocaproic acid (Bender & Krebs, 1950). Of the three branched amino acids tested L-leucine was the only one oxidized; this, too, is a similarity between the *Mytilus* and the cobra-venom enzyme. However, the cobra-venom enzyme oxidizes neither ornithine nor lysine.

The L-amino acid oxidase of *Neurospora crassa* studied by Bender & Krebs (1950) and by Thayer & Horowitz (1951) is in many ways similar to the *Mytilus* enzyme; it also acts on basic amino acids, including canavanine. However, there are differences: L-isoleucine is oxidized by the *Neurospora* enzyme.

Only one of the dicarboxylic diamino acids which did not contain sulphur was oxidized; this is αα′-diaminopimelic acid. This amino acid is known to be also a substrate of the L-amino acid oxidases of both *Neurospora crassa* and snake venoms (Work, 1955). Of the sulphur-containing amino acids of this group, all (cystine, homocystine, djenkolic acid and cystathionine) were found to be oxidized. L-Cystathionine is also oxidized by the *Neurospora* enzyme (Thayer & Horowitz, 1951).

The experiments reported raise the question as to the functional significance of the L-amino acid oxidase in *Mytilus*. It seems possible that in this species oxidative deamination may be an important pathway of degrading L-amino acids. However, as the digestive gland of *Mytilus* is in such intimate connexion with the lumen of the alimentary tract, the possibility that the oxidase is a bacterial enzyme cannot be fully excluded; Florkin (1952) has suggested that the cellulase found in molluscs is a bacterial enzyme.

In connexion with the localization of the *Mytilus* oxidase, it is of interest that the enzymic activity is so readily spun down by centrifuging. This is a property in which the *Mytilus* enzyme differs from all other amino acid oxidases hitherto described.

SUMMARY

1. The amino acid oxidase of the digestive gland of *Mytilus edulis* has been studied; it is shown that this enzyme acts upon L-amino acids.

2. The study of the substrate specificity of this enzyme shows that many amino acids are oxidized.

3. Earlier observations on the occurrence of a D-amino acid oxidase in *Helix aspersa* are confirmed.

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Studies in Detoxication

68. THE METABOLISM OF [^{14}C]NITROBENZENE IN THE RABBIT AND GUINEA PIG*

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In the previous paper on the metabolism of nitrobenzene in this series (Robinson, Smith & Williams, 1951), about 50% of an oral dose of nitrobenzene was accounted for as urinary metabolites. However, only the *p*-aminophenol and total nitro compounds excreted could be accurately determined, the amounts of the other metabolites formed being approximately assessed by paper chromatography and isolation procedures. Using nitrobenzene randomly labelled with ^{14}C in one carbon atom, it has now become possible to estimate accurately all the previously known metabolites, to demonstrate the existence of and identify the mercapturic acid suspected by Robinson *et al.* (1951) as a metabolite of nitrobenzene, and to show the formation of two new minor metabolites, namely carbon dioxide and nitroquinol. It has also been possible to measure the elimination of metabolites such as carbon dioxide, aniline and unchanged nitrobenzene in the expired air of the animal and to determine the amount eliminated in the faeces or retained in the tissues. It will be shown that some 85–90% of a single dose of nitrobenzene can now be accounted for.

MATERIALS AND METHODS

Melting points are corrected.

Preparation of [^{14}C]*nitrobenzene.* [^{14}C]Benzene (5 g.; Radiochemical Centre, Amersham) was added in small amounts to a mixture of HNO_3 (10 g.) and H_2SO_4 (15 g.), keeping the temperature below 45°. After keeping the mixture for 0·5 hr. at 0° the nitrobenzene layer was separated, and the remaining acid layer was diluted to 100 ml. with water and extracted with ether (2 × 10 ml.). The extract was added to the nitrobenzene and the mixture was washed with 10 ml. of water and then twice with 10 ml. of 10% (w/v) aqueous NH_4Cl. After drying over anhydrous $CaCl_2$, the ether was evaporated and the nitrobenzene distilled, b.p. 208–211° (yield 6·4 g., 69%). By isotopic dilution experiments it was shown that the non-volatile residue contained 24·5% of the radioactive benzene as *m*-dinitrobenzene, 2·4% as *o*-dinitrobenzene and 0·25% as *p*-dinitrobenzene.

Nitroquinol, m.p. 132° (Elbs, 1893); 3-nitrocatechol, m.p. 85°, and 4-nitrocatechol, m.p. 174° (Dakin, 1909); *o*-nitrothiophenol, m.p. 57° (Lecher & Simon, 1922); *m*-nitrothiophenol, m.p. 26°, and *p*-nitrothiophenol, m.p. 77° (Bennett & Berry, 1927); L-phenylmercapturic acid, m.p. 142° (Parke & Williams, 1951); and *trans-trans*-muconic acid, m.p. 300° (decomp.) (Ingold, 1921), were also prepared. Other known compounds required for isotope dilution experiments were purchased or prepared by standard methods and purified to constant m.p.

Animals. The [^{14}C]nitrobenzene contained about 100 μc/ml. of radioactivity, and was administered, alone or suitably diluted with inactive nitrobenzene (A.R.), b.p. 209–211°, to rabbits by stomach tube, and in one case to a guinea pig by intraperitoneal injection. After dosing, each rabbit was placed in a metabolism chamber through which a current of air was drawn into ethanol at −50° to trap any unchanged nitrobenzene or aniline, and thence into CO_2 absorbers (Parke & Williams, 1953). The expired CO_2 was collected only in Expts. 4 and 5 (see Table 1). In all the experiments the animals were removed from the chamber not later than 30 hr. after dosing.

* Part 67: Mead, Smith & Williams (1955).

In Expts. 4 and 5 the animals were killed and their tissues analysed for radioactivity. In the other experiments the animals were removed from the metabolism chamber after 30 hr. and placed in open cages so that their urine could be collected up to 10 days after dosing. The daily volume of urine was approximately 100 ml., and portions of 1–10 ml./day, according to the doses of ^{14}C, were taken for the isotopic dilution technique.

Measurement of radioactivity. Measurements were carried out on solid samples of 'infinite thickness' on nickel-plated iron planchets (General Electric Co. Ltd.) *in vacuo* (see Calvin, Heidelberger, Reid, Tolbert & Yankwich, 1949). In some experiments determinations of the total radioactivity were made by counting the solid residue obtained by evaporation of the urine under infrared lamps. These latter results did not differ significantly from those obtained by the combustion procedure.

The radioactivity of the tissues was measured as $BaCO_3$ or $CaCO_3$ obtained by wet combustion of the tissue dried on filter paper. The results for the various tissues are shown in Table 2, and Fig. 2 shows the rate of elimination of the radioactivity in the urine.

Table 1. *Elimination of unchanged nitrobenzene, aniline and* CO_2 *in the expired air of rabbits receiving* [^{14}C]*nitrobenzene orally*

Expt.	Dose of nitrobenzene (mg./kg.)	Dose of ^{14}C (μc/rabbit)	Duration of expt. (hr.)	Percentage of dose eliminated as		
				Nitrobenzene	Aniline	CO_2
3	250	0·6	24	0·7	0·04	—
5*	200	25	30	0·6	0	0·5
4	250	35	30	0·6	0	1·2

* This animal died after 30 hr. and was found to have large fat depots.

Table 2. *Occurrence of radioactivity in the tissues of rabbits receiving* [^{14}C]*nitrobenzene*

	Expt. 4 (8 days after dosing)*			Expt. 5 (1·5 days after dosing)*		
	Wt. of wet tissue (g.)	Specific activity of tissue (μc/g.)	% of dose present in tissue	Wt. of wet tissue (g.)	Specific activity of tissue (μc/g.)	% of dose present in tissue
Lungs	16	0·001	<0·1	18	0·003	0·2
Liver	46	0·001	0·1	52	0·004	0·8
Kidney	17	0·001	<0·1	18	0·050	3·6†
Heart	—	—	—	8	0·003	0·1
Spleen	—	—	—	2	0·004	0·05
Voluntary muscle	600	0·001	1·8	750	0·004	12·0
Brain	—	—	—	30	0·006	0·7
Fat (intestinal)	250	0·004	3·0	290	0·001	11·6
Fat (kidney)	210	0·004	2·4	230	0·017	15·4
Stomach contents	58	0·003	0·5	75	0·010	3·0
Intestinal contents (caecum)	36	0·004	0·4	42	0·018	3·1
Intestinal contents (small intestine)	—	—	—	72	0·010	2·9
Faeces	—	—	11·3	22	0·030	2·7
Total in tissues and faeces	—	—	19·6	—	—	56·2
Total in urine and expired air	—	—	58·8	—	—	24·1
Total accounted for	—	—	78	—	—	80

* No. of days after dosing with nitrobenzene when the animal was killed.
† This high figure is due to the high level of urinary excretion at the time of death.

using an end-window counter tube, the background of which was 9–12 counts/min. The specific activities were determined by comparison with a stable polymer reference (Radiochemical Centre, Amersham), which was standardized against ^{14}C-labelled *m*-dinitrobenzene, prepared from the labelled nitrobenzene. A sample of 1·75 sq.cm. containing 1 μc of ^{14}C/g. of substance gave approximately 1150 counts/min. The variation in disk size and preparation of sample gave an error of $\pm 5\%$.

Urine and tissues. The total radioactivity in the urine was measured as $BaCO_3$ obtained by wet combustion of the residue formed by evaporation of the urine over P_2O_5

ESTIMATION OF METABOLITES

Carbon dioxide. Each 4 hr. fraction of the CO_2 absorbed in the Drechsel bottles containing NaOH was converted into $BaCO_3$ and the radioactivity measured as described by Calvin *et al.* (1949).

Nitrobenzene and aniline in the expired air. The nitrobenzene and aniline eliminated in the expired air were determined spectrophotometrically in three experiments (cf. Azouz, Parke & Williams, 1952), using a Unicam spectrophotometer SP. 500. For the estimations the selected u.v. absorption maximum of nitrobenzene in

ethanol was at 260 mμ. where ε was 10 400 (slit width of 0·2 mm.), and that of aniline in ethanol was at 233 mμ. where ε was 11 500 (slit width of 0·5 mm.). It is unlikely that any metabolite, other than nitrobenzene, aniline or CO_2, was eliminated in the expired air, since in all experiments the u.v. absorption spectra of the ethanolic solutions from the absorption train showed no maxima other than those corresponding to nitrobenzene and aniline. Nitrobenzene was excreted in the expired air in all the experiments, to the extent of about 0·6% of the dose, but aniline was detected in only one experiment and amounted to 0·04% of the dose.

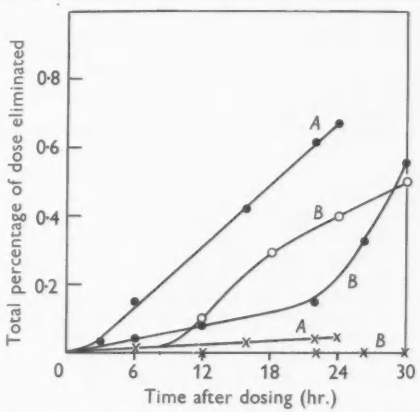

Fig. 1. Excretion of nitrobenzene (●), aniline (×), and CO_2 (○), in the expired air of rabbits receiving [^{14}C]-nitrobenzene orally. *A*, Expt. 3 (nitrobenzene, 0·25 g./kg.); *B*, Expt. 5 (nitrobenzene, 0·2 g./kg.).

The figures for the elimination of unchanged nitrobenzene, aniline, and CO_2 in the expired air are given in Table 1. Fig. 1 shows graphically the rate of elimination of nitrobenzene and aniline in Expts. 3 and 5. In none of the experiments was the expired air examined for longer than 30 hr. because of distress of the experimental animals. From the curves it is clear that the exhalation of unchanged nitrobenzene at 30 hr. after dosing was still at its maximal rate, so that the total nitrobenzene eliminated in the expired air might amount to 1–2% of the dose. The elimination of CO_2 in the expired air, which begins about 12–20 hr. after dosing, was also at its maximal rate at the end of the experiments.

Nitrobenzene and aniline in the urine. Nitrobenzene (100 mg.) and aniline (100 mg.) were added to one-half of the urine immediately it was passed, and were recovered diluted with the labelled materials by steam distillation of the urine made alkaline with Na_2CO_3 (pH 9). The aqueous distillate was acidified with 2N-H_2SO_4 and distilled to remove the nitrobenzene. Then, after addition of an excess of 2N-NaOH, the non-volatile residue was distilled to remove the aniline. The aqueous distillate containing the nitrobenzene was treated with an excess of $Ti_2(SO_4)_3$ (15%, w/v, in 23%, w/v, H_2SO_4) and the aniline so formed was converted into 2:4:6-tribromoaniline by addition of an excess of a saturated aqueous solution of bromine. The product was filtered, and recrystallized from ethanol to constant specific activity (m.p. and mixed m.p. 120°). The aqueous distillate containing the aniline was likewise treated with bromine water and the 2:4:6-tribromoaniline was recrystallized from ethanol to constant specific activity (m.p. 121°).

Phenols

Nitrophenols. *o*-, *m*- and *p*-Nitrophenols (100 mg.) were added to separate portions of the urine, which were then refluxed with 0·5 vol. of conc. HCl for 3 hr. to hydrolyse the phenolic conjugates. The solution containing added *o*-nitrophenol was then steam-distilled and the *o*-nitrophenol in the distillate was collected by filtration and dried over P_2O_5. The radioactivity of the *o*-nitrophenol (m.p. 45°) was determined, and the material was then dissolved in 5 ml. of 2N-NaOH and treated with 0·2 ml. of benzoyl chloride. The product was poured into water and the precipitated benzoate collected and recrystallized from aqueous ethanol. The *o*-nitrophenyl benzoate was further recrystallized from ethanol and methanol until its specific activity was constant (m.p. and mixed m.p. 59°).

The solutions containing the added *m*- and *p*-nitrophenols were separately extracted with ether continuously for 3 hr. The ether extracts were washed repeatedly with N-H_2SO_4 to remove aminophenols, and finally washed with water and evaporated to dryness. The residues were dissolved in NaOH and treated with benzoyl chloride, as above, to yield *m*-nitrophenyl benzoate, m.p. and mixed m.p. 95°, and *p*-nitrophenyl benzoate, m.p. and mixed m.p. 143° respectively, after recrystallization from ethanol and were further recrystallized from ethanol, methanol and light petroleum (b.p. 60–80°) to constant specific activity.

Aminophenols. *o*- and *m*-Aminophenols (200 mg.) and *p*-aminophenol sulphate (300 mg.) were added to separate portions of the urine, which were then refluxed with an equal vol. of conc. HCl for 5 hr. The solutions were cooled, adjusted to pH 8 with solid $NaHCO_3$, and continuously extracted with ether for 3 hr. The ethereal extracts were washed with water, and the aminophenols were transferred into 50 ml. of 2N-H_2SO_4. The acid solutions were washed with ether to remove nitrophenols, and then after adjusting to pH 8 with solid $NaHCO_3$ were extracted twice with 20 ml. portions of ether. The ethereal extracts were evaporated to dryness, and the residual aminophenols were benzoylated to yield the *O,N*-dibenzoyl derivatives of *o*-, *m*-, and *p*-aminophenols (m.p.'s and mixed m.p.'s 183, 153 and 237° respectively), after recrystallization from ethanol. These benzoyl derivatives were then further recrystallized successively from ethanol, ethyl acetate, light petroleum (b.p. 60–80°), and a mixture of $CHCl_3$ and CCl_4, to constant specific activity. In some experiments, in addition to the preparation of the dibenzoyl derivatives, the aminophenols were converted into the corresponding acetamidophenols by warming with acetic anhydride. The products were poured into a little water, and recrystallized from hot water to give *o*-, *m*-, and *p*-acetamidophenols (m.p. and mixed m.p. 207°, 149° and 169° respectively), which were further recrystallized from water and aqueous ethanol to constant specific activity.

Nitrocatechols. 3- and 4-Nitrocatechols (100 mg.) were added to separate portions of the urine which were then refluxed with 0·5 vol. of conc. HCl for 3 hr., cooled, and continuously extracted with ether for 4 hr. The ethereal extracts were washed with 2N-H_2SO_4 and then with water,

and finally were evaporated to dryness. The residue of 4-nitrocatechol was dissolved in 5 ml. of water and treated with 5 ml. of 10% (w/v) $BaCl_2$ solution to precipitate the barium salt. This material was purified by dissolving in 10 ml. N-HCl, treating with 5 ml. of 10% (w/v) $BaCl_2$ solution and reprecipitating the barium salt by adjusting to pH 7 with 2N ammonia. The barium salt was removed by filtration, dried *in vacuo* and its activity determined. The barium 4-nitrocatecholate was then dissolved in N-HCl, and the 4-nitrocatechol was extracted into ether, transferred into 2N-NaOH and treated with benzoyl chloride to yield 4-nitrocatechol dibenzoate (m.p. and mixed m.p. 156°) after recrystallization from aqueous ethanol. The activity of this material was then measured.

The residue of 3-nitrocatechol was dissolved in 5 ml. of water and treated with 10 ml. of lead acetate solution (3%, w/v) to precipitate the nitrocatechol as its lead salt. This was filtered off, suspended in 20 ml. of water and saturated with H_2S to remove the lead. After filtration, the 3-nitrocatechol was extracted from the solution by continuous extraction with ether for 3 hr. The ethereal extract was then evaporated to dryness, and the residue extracted with light petroleum (b.p. 60–80°) in which 4-nitrocatechol is insoluble. The soluble material was recrystallized from light petroleum (b.p. 60–80°) to give 3-nitrocatechol (m.p. and mixed m.p. 85°), which showed no radioactivity.

Nitroquinol. Nitroquinol (100 mg.) was added to a portion of the urine which was then refluxed with 0·5 vol. of conc. HCl for 3 hr., cooled, and twice extracted with ether. The ether extracts were washed with 2N-H_2SO_4 and water, dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue on recrystallization from CCl_4 gave nitroquinol (m.p. 131°), and its specific radioactivity was determined. This material was then benzoylated to give nitroquinol dibenzoate (m.p. and mixed m.p. 142°), which was recrystallized from ethanol to constant specific radioactivity.

The results on phenols and other aromatic metabolites are given in Table 3.

Table 3. *Elimination of phenols and other aromatic compounds in the urine of animals receiving* [^{14}C]*nitrobenzene*

The nitrobenzene was given orally to rabbits, except in Expt. 7, when it was administered to a guinea pig by intraperitoneal injection.

	Rabbits						Guinea pig
Expt.	1	2*	3	4	5†	6‡	7
Dose of nitrobenzene (mg./kg.) ...	250	400	250	250	200	200	500
Dose of ^{14}C (μc/animal)	0·6	1·0	0·6	35	25	25	18
Duration of expt. (days)	10	2	7	8	1·5	5	4
	Percentage of dose						
Nitrobenzene	<0·05	<0·05	0·05	<0·05	—	—	—
Aniline	0·2	0·3	0·2	0·6	—	—	—
o-Nitrophenol	0·1	<0·1	0·2	0·05	0·1	0·1	0
m-Nitrophenol	10·5	4·6	8·0	5·7	2·3	10·0	1·2
p-Nitrophenol	11·2	4·8	6·4	7·0	3·4	10·5	10·0
o-Aminophenol	1·5	3·5	3·8	3·1	3·4	3·1	3·6
m-Aminophenol	—	7·7	4·0	2·2	2·1	2·9	7·5
p-Aminophenol	33	26	23	33	8·4	33	26
3-Nitrocatechol	—	—	—	0	—	0	—
4-Nitrocatechol	0·9	0·4	0·2	0·5	0·4	1·0	0·5
Nitroquinol	—	—	—	0·1	0·05	0·05	0·1
o-Nitrophenylmercapturic acid	—	—	—	0	0	0	—
m-Nitrophenylmercapturic acid	—	—	—	0	0	0	—
p-Nitrophenylmercapturic acid	—	—	—	0·1	0·1	0·5	0·1
Nitrosobenzene	—	—	—	0	—	—	—
Azoxybenzene	—	—	—	0	0	—	0
Azobenzene	—	—	—	0	0	—	0
Benzidine	—	—	—	—	0	0	—
Phenol	—	—	—	0	0	—	0
Catechol	—	—	—	<0·1	<0·05	0	<0·05
Phenylmercapturic acid	—	—	—	0	—	0	0
trans-trans-Muconic acid	—	—	—	0	—	0	0
Sum of aromatic compounds	58	48	46	53	20	61	50
Total radioactivity of urine	68	41	45	57	23	63	58

* This animal died after 2 days. † This animal died after 30 hr., and was found to have abnormally large fat depots.
‡ This animal littered on the third day after dosing.

Mercapturic acids

The mercapturic acid excretion of rabbits dosed with nitrobenzene (150 mg./kg.) was estimated by Robinson *et al.* (1951) by I_2 titration, and was found to amount to about 3% of the dose, which was within the margin of error of the method. This iodometric estimation was repeated on the whole urine from two rabbits which had been fed with nitrobenzene (200 mg./kg.), and amounted in 2 days to 0·4 and 0·6±0·2% of the dose. The existence and identity

of the mercapturic acid were therefore investigated by the isotopic dilution technique, using *o*-, *m*-, and *p*-nitrothiophenols, since it was assumed that if any nitrophenylmercapturic acid were present it could be hydrolysed by alkali to the corresponding nitrothiophenol.

Nitrothiophenols. *o*-, *m*-, and *p*-Nitrothiophenols were separately added to portions of the urine and heated with 0·2 vol. of 10N-NaOH on a sand bath (120°) for 40 min. In the case of the *o*- and *p*-nitrothiophenols, the hydrolysed solutions were cooled, acidified with 2N-H_2SO_4 and continuously extracted with ether for 3 hr. The ethereal solutions were dried over anhydrous Na_2SO_4 and evaporated to dryness. The residues were dissolved in ethanol and treated with an excess of 5% (w/v) ethanolic I_2 to convert the thiophenols into the corresponding diphenyl disulphides. The resulting mixtures were diluted with water and the precipitated disulphides were filtered off, washed with water and recrystallized from benzene to yield 2:2′- and 4:4′-dinitrodiphenyl disulphides (m.p. and mixed m.p. 198° and 181° respectively). These derivatives were then further recrystallized from mixtures of benzene–ethanol and ethanol–ethyl acetate to constant specific activity.

The hydrolysed urine containing the *m*-nitrothiophenol was acidified with 2N-H_2SO_4 and was then steam-distilled. The *m*-nitrothiophenol in the distillate was collected into ether and treated with an excess of ethanolic I_2 (5%, w/v), and the resulting mixture was diluted with water. The precipitated dinitrodiphenyl disulphide was filtered off, washed with water and recrystallized from benzene to give 3:3′-dinitrodiphenyl disulphide (m.p. and mixed m.p. 83°), which on further recrystallization from ethanol–ethyl acetate showed no radioactivity.

Of the added nitrothiophenols, only the *para*-isomer was found to entrain radioactivity. From these results it is deduced that *p*-nitrophenylmercapturic acid is a minor metabolite of nitrobenzene, equivalent to about 0·3% of the dose.

Reduction products

Robinson *et al.* (1951) suggested that most of the *p*-aminophenol which results from the metabolism of nitrobenzene is derived via phenylhydroxylamine. The urines were therefore examined for the presence of nitrosobenzene which is an atmospheric oxidation product of *β*-phenylhydroxylamine and a possible intermediate in the biological formation of phenylhydroxylamine from nitrobenzene. In the presence of nitrosobenzene, phenylhydroxylamine *in vitro* gives rise to azoxy, azo- and hydrazo-benzene. Hydrazobenzene is also a product of the metabolism of azobenzene in the rat and was isolated from the acid-treated urine as benzidine (Elson & Warren, 1944). The urines were therefore also examined for the presence of azoxybenzene, azobenzene and benzidine.

Nitrosobenzene. Nitrosobenzene (100 mg.) was added to a portion of the urine, which was refluxed with 0·5 vol. of conc. HCl for 3 hr. and then steam-distilled. The nitrosobenzene was collected from the steam distillate into ether, and the ethereal solution was washed with N-NaOH and then water to remove any *o*-nitrophenol. After drying over anhydrous Na_2SO_4, the ethereal solution was evaporated to dryness and the residue of nitrosobenzene was repeatedly recrystallized from aqueous ethanol. The final product (m.p. 68°) contained no radioactivity.

Azoxybenzene. Azoxybenzene (100 mg.) was added to a portion of the urine, which was then refluxed with 0·5 vol. of conc. HCl for 3 hr. The solution was cooled and continuously extracted with ether for 4 hr. The ether extract was washed several times with N-NaOH to remove phenolic materials, and was then evaporated to dryness. The residue of crude azoxybenzene was recrystallized repeatedly from aqueous ethanol and the final product (m.p. 36°) contained no radioactivity.

Azobenzene. A portion of the urine (10 ml.) was shaken for 2 hr. with 100 mg. of azobenzene dissolved in 10 ml. of ether. The ether layer was separated and was washed with 2N-NaOH to remove nitrophenols, with 2N-H_2SO_4 to remove aminophenols, and finally with water. The ethereal solution was then dried over anhydrous Na_2SO_4 and evaporated to dryness. The residue of azobenzene was recrystallized several times from ethanol and from light petroleum (b.p. 60–80°) and the final product (m.p. 68°) showed no radioactivity.

Benzidine. Benzidine (100 mg.) was added to a portion of urine which was refluxed for 3 hr. with 0·5 vol. of conc. HCl. The solution was cooled and treated with an excess of 2N-H_2SO_4 to precipitate the benzidine sulphate, which was removed by centrifuging, and washed with water, ethanol, and finally ether. The benzidine sulphate was then dissolved in warm N-NaOH, filtered, and reprecipitated by addition of excess of 2N-H_2SO_4. This process was repeated several times, and the product, although still radioactive, showed a continuously decreasing specific activity. The benzidine sulphate was then treated with a slight excess of N-NaOH and the solution was continuously extracted with ether for 3 hr. The ethereal solution was dried over anhydrous Na_2SO_4, evaporated to dryness, and the residue of benzidine was recrystallized from aqueous ethanol. The product (m.p. 125°) was still slightly radioactive, and was therefore treated in solution in 2N-NaOH with benzoyl chloride to yield the dibenzoyl derivative (m.p. 350° decomp.), which was free from radioactivity.

Denitration products

The action of X-rays on dilute solutions of nitrobenzene is similar to the action of the free hydroxyl radicals of Fenton's reagent, but, in addition to the production of *o*-, *m*- and *p*-nitrophenols, the nitro group is eliminated with the formation of phenol (Loebl, Stein & Weiss, 1949, 1950). Furthermore, Bray, Hybs, James & Thorpe (1953) have shown that 2:3:5:6-tetrachloronitrobenzene is converted in the rabbit into L-2:3:5:6-tetrachlorophenylmercapturic acid, the nitro group of the original substance being eliminated. The metabolism of 2:3:4:6-tetrachloronitrobenzene and pentachloronitrobenzene into L-2:3:4:6-tetrachlorophenyl- and L-pentachlorophenyl-mercapturic acids by the rabbit are two further examples of conjugation with acetylcysteine with the concomitant elimination of an aromatic nitro group (Betts, James & Thorpe, 1953). It was therefore necessary to ascertain whether denitration of nitrobenzene to benzene or its metabolites could occur in the animal body, and accordingly the rabbit urines were examined for phenol, and for catechol, *trans-trans*-muconic acid and L-phenylmercapturic acid, which are metabolites of benzene which may not arise via phenol (Parke & Williams, 1953).

Phenol and catechol. These phenols (100 mg. each) were added to separate portions of the urine which were subsequently hydrolysed by refluxing with 0·5 vol. of conc. HCl for 3 hr. The phenol and catechol were then isolated by the

procedures described by Parke & Williams (1953) and their toluene-*p*-sulphonates were found to contain no radioactivity.

Phenylmercapturic acid. Synthetic L-phenylmercapturic acid (200 mg.) was added to a portion of the urine, which was then heated with 0·2 vol. of 10N-NaOH on a sand bath (120°) for 40 min. The hydrolysed urine was acidified with 2N-H_2SO_4 and steam-distilled. The thiophenol in the distillate was collected into ether and treated with an excess of 5% (w/v) ethanolic I_2, and the product diluted with water. The precipitate of diphenyl disulphide was filtered off and after repeated recrystallization from ethanol (m.p. 61°) was found to contain no radioactivity.

trans-trans-*Muconic acid.* This carboxylic acid oxidation product of benzene was estimated by the isotopic dilution procedure described by Parke & Williams (1953), and was shown not to occur in nitrobenzene urine.

RESULTS AND DISCUSSION

The fate of an oral dose of nitrobenzene administered to rabbits has now been accounted for within the limits of the experimental methods, and is shown in summary in Table 4. During 4–5 days after dosing with [^{14}C]nitrobenzene some 70% of the radioactivity is eliminated from the body in the expired air, urine and faeces. The rest remains in the body, from which it is slowly excreted in the urine and probably also in the expired air as carbon dioxide. The urinary excretion of metabolites is almost complete within 5 days of dosing, but the urines are still weakly radioactive even after 10 days (see Fig. 2). The radioactivity remaining in the body in Expt. 4, when the animal was killed 8 days after dosing, was probably present entirely as metabolites, since no odour of nitrobenzene was detectable in the tissues. In Expt. 5, however, where the animal died 30 hr. after dosing, the odour of nitrobenzene in the tissues was pronounced, particularly in the fat depots, which were abnormally large in this animal. This confirms the findings of Robinson *et al.* (1951) that the fat of a rabbit killed 2 days after dosing contained small amounts of unchanged nitrobenzene. The radioactivity in the body was located principally in the fat, but apart from this it was equally distributed among the other tissues.

Only a very minor fraction of the material was eliminated from the body in the expired air. About 0·5% of the dose was exhaled as unchanged nitrobenzene, and in one case only (Expt. 3) a trace of aniline (0·04% of the dose) was also found. Respiratory carbon dioxide amounting to about 1% of the dose was excreted in 30 hr., but it was unlikely that elimination of this metabolite was complete in this time.

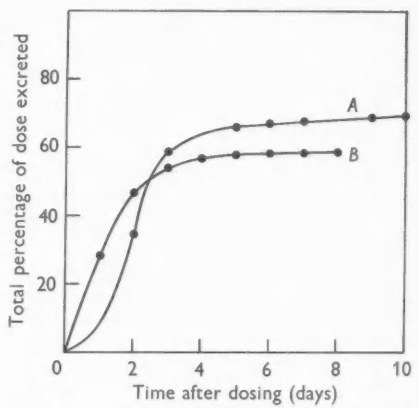

Fig. 2. Excretion of radioactivity in the urine of rabbits receiving [^{14}C]nitrobenzene orally (dose, 0·25 g./kg.). *A* is Expt. 1 and *B* is Expt. 4.

Table 4. *Metabolic fate of a single oral dose of* [^{14}C]*nitrobenzene in the rabbit during 4–5 days after dosing*

Metabolite	Percentage of dose (average)		
Respiratory CO_2	1		
Nitrobenzene	0·6*	2 in expired air	
Aniline	0·4†		
o-Nitrophenol	0·1		
m-Nitrophenol	9		
p-Nitrophenol	9		
o-Aminophenol	3	58 in urine	60 total
m-Aminophenol	4		
p-Aminophenol	31		
4-Nitrocatechol	0·7		
Nitroquinol	0·1		
p-Nitrophenylmercapturic acid	0·3		
(Total urinary radioactivity)	(58)		
Metabolized nitrobenzene in faeces	9‡		
Metabolized nitrobenzene in tissues	15–20		
Total accounted for	85–90%		

* 0·5% in the expired air and <0·1% in the urine.
† 0·3% in the urine and <0·1% in the expired air.
‡ 6% of the dose was present in the faeces as *p*-aminophenol.

About 58% of the dose is excreted in the urine, all of which is present as aromatic compounds. The major urinary metabolite of nitrobenzene is *p*-aminophenol, which accounts for 31% of the dose, or more than half of the nitrobenzene metabolized. *m*- and *p*-Nitrophenols are the next major metabolites, each accounting for 9% of the dose, and then *m*- and *o*-aminophenols, which amount to 4 and 3% respectively. 4-Nitrocatechol (0·7%), aniline (0·3%), *o*-nitrophenol (0·1%), and nitroquinol (0·1%) are minor metabolites, nitroquinol being a hitherto-undescribed metabolite of nitrobenzene. 3-Nitrocatechol was not found to be a metabolite. Apart from aniline and these phenolic compounds, the presence of unchanged nitrobenzene in the urine in amounts of less than 0·1% of the dose (Robinson *et al.* 1951) was confirmed. These quantitative figures are in very good agreement with those obtained by Robinson *et al.* (1951) with unlabelled nitrobenzene (see Table 5).

Table 5. *Comparison of quantitative estimations of the urinary metabolites of nitrobenzene in the rabbit, using non-radioactive and radioactive nitrobenzene*

Metabolites	With non-radioactive nitrobenzene* (Robinson *et al.* 1951) (% of dose)	With radioactive nitrobenzene† (this paper) (% of dose)
Nitrobenzene	0·03	< 0·1
o-Nitrophenol	0·05	0·1
m-Nitrophenol	4·0	9
p-Nitrophenol	5·0	9
4-Nitrocatechol	0·5	0·7
Nitroquinol	—	0·1
Total nitro compounds	20‡	18·9
Aniline	0·50	0·3
o-Aminophenol	0·54	3
m-Aminophenol	0·58	4
p-Aminophenol	35·0	31
Total metabolites	56	57

* Urine collected for 2 days after dosing.
† Urine collected for 4–5 days after dosing.
‡ Estimated by $TiCl_3$ titration.

The mercapturic acid was identified as *p*-nitrophenylmercapturic acid, and was estimated to be equivalent to about 0·3% of the dose. This is considerably less than the estimate of 3% of Robinson *et al.* (1951), but more in agreement with the figure of 0·5% obtained in the present paper when the whole of the urine was used for the iodometric titration.

L-Phenylmercapturic acid, which could have arisen by conjugation with acetylcysteine with concomitant displacement of the nitro group (see Bray *et al.* 1953), was found not to be a metabolite of nitrobenzene. This result, together with the absence from the urine of phenol, catechol and *trans-trans*-muconic acid, shows that elimination of the nitro group does not occur while the aromatic ring is still intact. Since carbon dioxide is a metabolite of nitrobenzene, the elimination of the nitro group must occur either during the opening of the ring, when the product of fission is other than *trans-trans*-muconic acid, or at some subsequent stage of degradation of this fission product.

As nitrosobenzene and β-phenylhydroxylamine are probably intermediates in the formation of *p*-aminophenol, the major metabolite of nitrobenzene, and are undoubtedly also the precursors of aniline (Robinson *et al.* 1951), it seemed reasonable to expect to find evidence of the existence of these intermediate compounds, as traces of azoxy-, azo- or hydrazo-benzene, in the urine. Since azobenzene is metabolized by the rabbit into aniline and hydrazobenzene (Elson & Warren, 1944), and the latter is converted into benzidine by treatment with strong acids, the most likely evidence to be found in the urine of the intermediate stages of reduction would appear to be benzidine. Since none of these compounds were found in the urine, the stepwise reduction of nitrobenzene to nitrosobenzene and β-phenylhydroxylamine, and the rearrangement to *p*-aminophenol, *in vivo*, remain only conjectural.

In the single experiment in which the [^{14}C]-nitrobenzene was administered to a guinea pig, the only marked differences between this animal and the rabbits were the complete absence of *o*-nitrophenol and the decrease of *m*-nitrophenol in the urine of the guinea pig. It is possible that the *o*- and *m*-nitrophenols are more readily reduced to the corresponding aminophenols in the guinea pig than in the rabbit, since the total *ortho* and *meta* compounds (i.e. nitro plus amino) are approximately the same in comparative cases of both animals. Thus the sum of *o*-nitro- and *o*-aminophenols in Expt. 4 is 3·15% and in Expt. 7 is 3·6%; and the figures for the corresponding *meta* compounds are: Expt. 4, 7·9%; Expt. 7, 8·7%. The total amount of reduction is approximately constant however, the sum of the aminophenols in Expt. 4 being 38·3%, and in Expt. 7, 37·1%.

SUMMARY

1. A study has been made of the fate in rabbits of nitrobenzene labelled randomly in one carbon atom with ^{14}C.

2. In 30 hr. the expired air contains about 1% of the dose as carbon dioxide and 0·6% as unchanged nitrobenzene. The elimination of carbon dioxide is not complete within this time.

3. In 4–5 days, 58% of the nitrobenzene is eliminated in the urine. This is present as the previously established metabolites: *p*-aminophenol

(31%), *m*-nitrophenol (9%), *p*-nitrophenol (9%), *m*-aminophenol (4%), *o*-aminophenol (3%), 4-nitrocatechol (0·7%), aniline (0·3%) and *o*-nitrophenol (0·1%). Two new metabolites, nitroquinol (0·1%) and *p*-nitrophenylmercapturic acid (0·3%), were found in the urine.

4. The denitration products, phenol, catechol, L-phenylmercapturic acid, and *trans-trans*-muconic acid, did not occur in the urine.

5. Nitrosobenzene, azoxybenzene, azobenzene and benzidine, the presence of which would have substantiated the occurrence of nitrosobenzene and phenylhydroxylamine as intermediates in the formation of *p*-aminophenol, were not detected in the urine.

6. The total excretion of radioactivity in the expired air, urine and faeces accounts for nearly 70% of the dose of nitrobenzene in 4–5 days after dosing. The remainder is present in the tissues and is eliminated slowly as metabolites in the urine, which is still radioactive 10 days after dosing, and probably also as carbon dioxide.

7. Two days after dosing, about 54% of the administered radioactivity was found in the tissues, particularly in the fat and the intestinal tract. Unchanged nitrobenzene was present in the tissues of this animal. Eight days after dosing, in another experiment, 8% of the radioactivity was found in the tissues, the fat being the most active. No free nitrobenzene was detected in this animal.

8. The faeces contain about 9% of the administered radioactivity, of which about 6% is present as *p*-aminophenol.

9. These experiments account for 85–90% of the administered nitrobenzene.

I am indebted to Professor R. T. Williams for his constant interest in this work. The expenses of this work were in part defrayed by a grant to Professor Williams from the Medical Research Council.

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Studies in Carotenogenesis

19. A SURVEY OF THE POLYENES IN A NUMBER OF RIPE BERRIES*

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During the past few years a number of new C_{40} polyenes have been found in plants (Karrer & Jucker, 1950; Goodwin, 1952*a*, 1955*a*). Perhaps the most important of these from the viewpoint of carotenogenesis is the series of hydrocarbons, chemically related to lycopene, consisting of tetrahydrophytoene, phytofluene, ζ-carotene, neurosporene and lycopene. (The first three members of this series are colourless; hence the term 'carotene' will be used only in discussing the coloured members of the series, and the term 'polyene' will be used as a general term including both coloured and colourless members.) As a result of a study of the distribution of these polyenes in the fruit of various tomato crosses, Porter & Lincoln (1950) concluded that the biosynthesis of lycopene probably occurred via this series by sequential dehydrogenation of the almost completely saturated tetrahydrophytoene.

One of the aims of the present survey was to discover, within the limits of the species available, how widespread was the distribution of the 'Porter–Lincoln' series in fruit and berries.

Goodwin (1952*a*, 1955*a*, *b*) lists the polyenes found in the fruit of some 100 plant species. Most of these species, however, were investigated from

* Part 18: Goodwin & Jamikorn (1956).

a chemical point of view, and only the major components were isolated and examined; the minor components, which, from the trend of modern developments in carotenoid biochemistry, might be of considerable importance, were rarely examined. The second aim of this investigation was, therefore, to begin the accumulation of information on all polyenes occurring in various fruit and berries. It is interesting that since the experimental work reported here was completed, a number of workers have concerned themselves with the distribution of the minor components of carotenoids in plants materials (e.g. Haxo, 1952; Turian & Haxo, 1954; Taha, 1954; Curl & Bailey, 1954).

Twenty-three species, representing eight families, have been examined, most of them for the first time. In the few species which have been re-examined, the minor components have not been considered previously.

EXPERIMENTAL

Materials. The species investigated, together with their trivial names and the localities where they were collected, are listed in Table 1. All specimens were collected during August–September 1952; a second sample of *Arum maculatum* was collected during the same period of 1953.

Extraction of polyenes. The crushed berries were ground to a fine dry powder with anhydrous Na_2SO_4 and acid-washed silver sand, and the pigments extracted in the cold by stirring with successive portions of diethyl ether until no further colour was removed; usually three to four treatments were sufficient. Each extract was filtered through a sintered-glass filter (G × 16) and the combined extracts were evaporated to dryness *in vacuo* at 25°. In order to remove neutral fat and chlorophyll, which might interfere with the chromatographic separation of the polyenes, the residue was saponified by boiling for 2 min. with a suitable volume (approx. 10 ml.) of 20% (w/v) ethanolic KOH. The unsaponifiable matter (containing the polyenes) was extracted by the method of Goodwin & Morton (1946) and dissolved in light petroleum (10–20 ml.) (light petroleum b.p. 40–60° was used throughout this investigation), ready for chromatographic separation. In some cases the extracts were first dissolved in a few drops of ethyl ether before addition of the light petroleum. This was necessary only when petroleum-insoluble xanthophylls were present.

Chromatographic separation of polyenes. The polyene mixtures were first separated into hydrocarbons and xanthophylls on a column of deactivated alumina, prepared by treating activated alumina (Grade 'O', P. Spence and Co., Widnes) with methanol (Goodwin & Srisukh, 1949). The hydrocarbon polyenes were removed with light petroleum containing 5% (v/v) of diethyl ether, and collected in the filtrate. The solvent was then removed *in vacuo* at 25–30°, the residue redissolved in light petroleum and separated into its components by chromatography on a 3:1 mixture of activated and deactivated alumina, light petroleum containing various amounts of diethyl ether as developer being used (Goodwin, 1952*b*). If any fractions overlapped on the chromatogram, they were rechromatographed under slightly different experimental conditions (e.g. with small variations in either the ratio of activated to deactivated alumina or the concentration of ether in the developer) until homogeneous. The polyenes were then identified by their position on the chromatogram relative to other known polyenes and by the shape and position of their absorption spectra. These are sufficient criteria for the common polyenes because: (*a*) considerable experience in this and other Laboratories (Haxo, 1952; Porter & Lincoln, 1950; Trombly & Porter, 1953; Turian & Haxo, 1954; Goodwin, 1952*b*, *c*, 1953*a*, 1954, 1955*b*) has shown that

Table 1. *Details of the various species of berries examined*

Scientific name	Family	Trivial name	Place collected
Arum maculatum	Araceae	Cuckoo Pint, Lords and Ladies	N. Wales, near Chester
Atropa belladonna	Solanaceae	Deadly Nightshade	N. Wales
Berberis barbarossa	Berberidaceae	Barberry	Somerset
Berberis sp. (Pirate King)	Berberidaceae	Barberry	Somerset
Capsicum annuum	Solanaceae	Red pepper	Bought dried from shop
Cotoneaster bullata	Rosaceae	Cotoneaster	Somerset
C. frigida	Rosaceae	Cotoneaster	Somerset
C. hebephylla	Rosaceae	Cotoneaster	Somerset
Crataegus oxyacantha	Rosaceae	Hawthorn	N. Wales
C. pratensis	Rosaceae	Hawthorn	N. Wales
Hippophaë rhamnoides	Elaeagnaceae	Sea buckthorn	Somerset
Lonicera periclymenum	Caprifoliaceae	Honeysuckle	N. Wales
*Pyracantha flava**	Rosaceae	Firethorn	Somerset
*P. rogersiana**	Rosaceae	Firethorn	Somerset
Rosa canina	Rosaceae	Rose (hips)	N.W. Cheshire
R. moyesii	Rosaceae	Rose (hips)	Somerset
R. rubrifolia	Rosaceae	Rose (hips)	N. Wales
Sambucus nigra	Caprifoliaceae	Elderberry	N. Wales
Solanum dulcamara	Solanaceae	Woody nightshade	N.W. Cheshire
Sorbus aucuparia	Rosaceae	Rowan; mountain ash	N. Wales
Tamus communis	Dioscoreaceae	Black bryony	N.W. Cheshire
Taxus baccata	Taxaceae	Yew	N.W. Cheshire
Viburnum opulus	Caprifoliaceae	Guelder rose	Somerset

* These are cultivars of *P. crenulata*.

they always separate on a column in the order given in Table 2; (*b*) the absorption spectra maxima of adjacently adsorbed polyenes are quite distinct and characteristic. Whenever unusual polyenes, such as *cis* isomers, have been observed these have been examined in greater detail.

Table 2. *Adsorption sequence and absorption maxima of the common hydrocarbon polyenes**

Adsorbent, Al_2O_3; polyenes in order of increasing adsorption. Chromatographic methods are given in the Experimental section.

Polyene	$E_{max.}$ in light petroleum (mμ.)
Phytoene	275, 285, 296
Phytofluene	332, 348, 367–368
α-Carotene	420, 443, 474
β-Carotene	424, 450, 481
ζ-Carotene	378, 400, 419
δ-Carotene	428, 458, 490
γ-Carotene	431, 461, 494
Neurosporene	410, 433, 468
Lycopene	445, 491, 504

* For a complete list in sequence see Goodwin (1955*b*).

Table 3. *Adsorption sequence and absorption maxima of the common xanthophylls*

Adsorbent, deactivated Al_2O_3; developer, light petroleum containing various concentrations of ethanol; xanthophylls in order of increasing adsorption.

Xanthophyll	$E_{max.}$ in light petroleum (mμ.)
Flavochrome, Mutatochrome	427, 456
Cryptoxanthin	420, 451, 482
Rubixanthin	432, 463, 495
Lycoxanthin	443, 471, 502
Rubichrome	472, 501*
†	
Lutein	418, 442, 477
Zeaxanthin	424, 451, 483
Chrysanthemaxanthin	421, 448
Flavoxanthin	421, 449
Lycophyll	443, 471, 502
Violaxanthin	442, 471
Neoxanthin	437, 466

* In CS_2.

† A considerable gap in the chromatogram occurs here because of the change from mono- to di-hydroxy carotenoids.

The xanthophyll fraction. The pigments remaining on the deactivated alumina after the removal of the hydrocarbon polyenes are fractionated by development with light petroleum containing increasing amounts of ethyl ether. Some of the more strongly adsorbed carotenoids are not eluted even with pure ether; in these cases development is continued with ethyl ether containing increasing amounts of ethanol. As with the hydrocarbons the well-described xanthophylls can be identified by their relative adsorptive properties and the shape and position of their absorption spectra, together with ancillary tests, such as partition between light petroleum and methanol containing either 5 or 10% (v/v) of water and interaction with HCl in ethereal solution.

Table 3 shows the relative positions, found in this Laboratory, of the commoner xanthophylls on a column of alumina. Contrary to the experience with the hydrocarbon polyenes, care must be taken not to extrapolate this order to other adsorbents, or even to alumina used with other developing solvents (Strain, 1948). Xanthophylls that are not included in Table 3 are considered in detail in the appropriate section.

Quantitative determination of the polyenes. Good estimates of the total carotenoid content of the crude extracts were made by measuring $E_{1\,cm.}$ at 450 mμ. of the extract and assuming $E^{1\%}_{1\,cm.}$ for the mixture to be 2500, the value for β-carotene. For these determinations, the presence of the colourless polyenes was ignored. The various purified fractions were dissolved in known volumes of light petroleum and the $E_{max.}$ values measured in a 1 cm. cell. By comparing these with the $E^{1\%}_{1\,cm.}$ values for the pure pigments (Karrer & Jucker, 1950; Goodwin, 1952*a*) the amounts of the pigments present could be calculated. If the $E^{1\%}_{1\,cm.}$ value of a pure pigment was not known, it was assumed to be 2500. No allowance was made for losses on chromatography; for the hydrocarbons these are negligible, but with the highly oxygenated xanthophylls appreciable losses, up to 25%, may occur.

Spectrophotometric measurements. These were carried out with either a Beckman DU or Unicam SP. 500 instrument.

RESULTS

Carotenoid concentration and relative amounts of hydrocarbons and xanthophylls in the berries

Table 4 shows that the concentration of carotenoids varies from almost zero (0·2 μg./g.) to 600 μg./g., and that the relative amounts of hydrocarbons also

Table 4. *Total carotenoid content and relative amounts of hydrocarbon polyenes in berries of various species*

Species	Carotenes (as % of total carotenoids)	Amount of carotenoids (μg./g. wet wt.)
Arum maculatum	92, 89	200
Atropa belladonna	23	18
Berberis barbarossa	—	0·25
Berberis sp. (Pirate King)	—	0·2
Capsicum annuum	35	127
Cotoneaster bullata	10	12
C. frigida	37	1·5
C. hebephylla	7	16
Crataegus oxyacantha	40, 41	4, 25
C. pratensis	57	5
Hippophaë rhamnoides	3	52
Lonicera periclymenum	15	18
Pyracantha flava	59, 27	9, 15
P. rogersiana	—	1
Rosa canina	26	25
R. moyesii	61	224
R. rubrifolia	49	88
Sambucus nigra	19	16
Solanum dulcamara	61	90
Sorbus aucuparia	39	120
Tamus communis	85	96
Taxus baccata	5	10*
Viburnum opulus	32·5	607

* Calculated on whole berry, not just the aril.

Table 5. *Qualitative and quantitative distribution of the common hydrocarbon polyenes in various berries*

A dash indicates the absence of a polyene; the failure of the percentages of the constituents to add up to 100 indicates the presence of the uncommon pigments discussed in the text.

Berries	Percentage of various components						
	Phytoene*	Phytofluene	β-Carotene	ζ-Carotene	γ-Carotene	Prolycopenes	Lycopene†
Arum maculatum‡	—, 0·4	3·8, 1·6	25·2, 7·9	0·3	0·2	39·1, 41·8	31·4, 49·3
Atropa belladonna	—	—	100	—	—	—	—
Berberis barbarossa	—	—	100	—	—	—	—
Berberis sp. (Pirate King)	—	—	100	—	—	—	—
Capsicum annuum	19·9	16·4	63·7	—	—	—	—
Cotoneaster bullata	—	Trace	100	Trace	—	—	—
C. frigida	—	—	100	—	—	—	—
C. hebephylla	—	—	100	—	—	—	—
Crataegus oxyacantha	—	—	100	—	—	—	—
C. pratensis	—	?Trace	100	Trace	—	—	—
Hippophaë rhamnoides	—	Trace	100	3·7	3·7	—	15·4
Lonicera japonica§	2·8	8·0	55·0	3·4	—	—	45·3
L. periclymenum	—	?Trace	51·3	—	—	—	—
Pyracantha flava‖	—	Trace, 2·1	90·8, 100	—	—	—	—
P. rogersiana	—	—	100	—	—	—	—
Rosa canina	Trace	1·4	66·3	0·9	5·3	2·2	24·9
R. moyesii	7·6	4·6	24·7	5·6	—	21·6	36·1
R. rubrifolia	5·0	5·3	57·2	Trace	?	—	32·5
Sambucus nigra	—	—	100	—	—	—	—
Solanum dulcamara	—	Trace	Trace	—	—	Trace	100
Sorbus aucuparia	—	—	100	Trace	—	—	—
Tamus communis	4·0	1·5	4·7	—	—	—	89·8
Taxus baccata	15·0	4·6	76·5	—	—	—	—
Viburnum opulus	—	13·9	4·1	8·0	8·9	—	56·2

* In many cases *trans*-phytofluene was also observed in traces. In view of the observation of Koe & Zechmeister (1953) that phytofluene is extremely heat-labile, it is probable that the traces of *trans*-phytofluene in our extracts were artifacts.
† Includes neolycopene. ‡ The two values are for specimens collected in different years.
§ Previous observations (Goodwin, 1952*c*) recorded here for comparison.
‖ The two values are for two samples of the same crop.

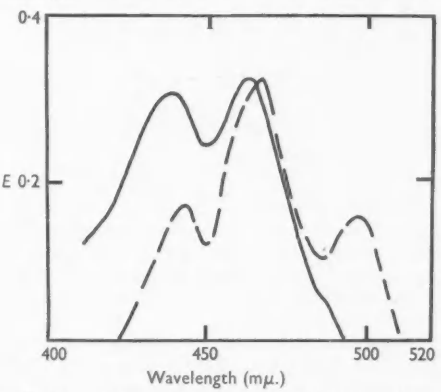

Fig. 1. Absorption spectra in light petroleum of prolycopene II before (full line) and after I_2 treatment (broken line). $E_{max.}$ has been adjusted to the same value for each pigment.

vary widely from 3 to 92%. These values must be taken only as a general guide, because the concentrations and relative amounts of the constituents, especially the former, can vary considerably with the degree of ripeness (Goodwin, 1952*a*). All the specimens examined were 'ripe', but the exact degree of ripeness was not known.

Constituent hydrocarbon polyenes

In order to save space the individual protocols for each species are not given; the occurrence and quantitative distribution of the well-characterized hydrocarbons are given in Table 5.

Prolycopenes. The first fractionations of the polyenes of *Arum maculatum* revealed a large brown zone between γ-carotene and lycopene. On rechromatographing this zone on active alumina, three fractions were obtained, with spectral maxima corresponding with those of prolycopenes I, II and III (Zechmeister & Pinckard, 1947). The shapes were not, however, completely identical with those of Zechmeister & Pinckard. This is probably due to the difficulty of obtaining them chromatographically homogeneous, but iodine isomerization convincingly demonstrated their prolycopene nature, for immediately on the addition of iodine all underwent a marked shift in absorption maxima to give the characteristic curve of the equilibrium mixture of *cis–trans*-lycopenes (Zechmeister, 1944); this is illustrated in Fig. 1 for the pigment considered to be prolycopene II.

Table 6. *Distribution of xanthophylls in various berries*

Amounts in per cent of total xanthophylls present;* a gap indicates the absence of a pigment. *Pyracantha rogersiana* and *Tamas communis* are not included, since only traces of unexamined xanthophylls were present.

Species	Mutatochrome	Cryptoxanthin	Zeaxanthin	Lutein	Flavoxanthin or chryxanthemaxanthin†	Others‡
Arum maculatum	—	Present	—	—	—	—
Atropa belladonna	—	25·0	—	—	—	—
Berberis spp.§	—	—	14·2	44·6	—	Capsanthin,‖ 43·3
Capsicum annuum	—	11·7	16·7	—	8·5	Capsorubin,‖ 17·3
Cotoneaster bullata	16·0	13·4	Trace	—	—	Aurochrome, 54·0
C. frigida	9·5	13·8	21·4	—	32·1	—
C. hebephylla¶	5·6	5·1	—	—	41·1	—
Crataegus oxyacantha	20·6	27·7	—	24·4	—	Auroxanthin, 14·9
C. pratensis	9·2	12·3	—	—	—	—
Hippophaë rhamnoides	—	Trace	57·1	13·9	—	Aurochrome, 2·1
*Lonicera japonica** ¶ **	—	77·9	4·2	6·6	—	Auroxanthin, 6·0
L. periclymenum	—	44·6	40·5	—	—	Auroxanthin, 13·0
Pyracantha flava	12·7	—	23·7	48·2	14·6	Luteochrome, trace
Rosa canina	—	2·3	8·1	—	—	Rubixanthin,‖ 56·5
R. moyesii	2·5	28·6	11·5	16·2††	Trace	Rubixanthin, 34·9
R. rubrifolia	—	7·9	5·6‡‡	—	4·5	Rubixanthin, 82·0
Sambucus nigra	—	Trace	—	76·8	11·2	Neoxanthin, 11·2
Solanum dulcamara	Trace (?)	—	—	70·4	—	Lycoxanthin, 29·6
Sorbus aucuparia	59·6	—	Trace	14·3	—	—
Taxus baccata	3·5	—	—	16·9	Trace	Rhodoxanthin, 78·6
Viburnum opulus	95	—	—	—	—	—

* Failure of the figures to add up to 100 indicates presence of pigments discussed further in text or indefinite oxidation products.

† It is very difficult to separate these stereoisomers (Karrer & Jucker, 1950); no attempt was made to do so in this survey.

‡ Additional pigments also probably present in traces but the total amounts of xanthophylls were too small to confirm this.

§ The same results in all species examined; only traces of xanthophylls were present.

‖ *Cis* isomers present.

¶ Data from Goodwin (1952*c*).

** Pigment X (Goodwin & Osman, 1953) also present.

†† This fraction was rather more strongly adsorbed than is usual for lutein; it might be lutein 5:6-epoxide.

‡‡ Also contains traces of lutein.

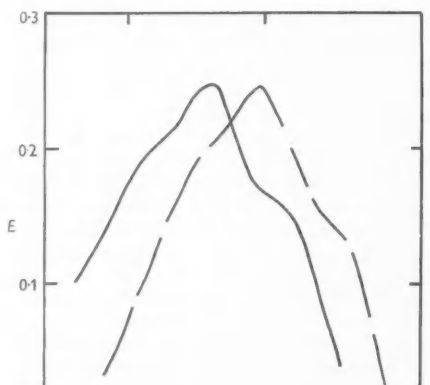

Fig. 2. Absorption spectra of rhodoxanthin in light petroleum (full line) and benzene (broken line). $E_{max.}$ has been adjusted to the same value for each pigment.

Prolycopenes, with prolycopene II predominating, were also observed in smaller amounts in *Rosa* spp. and *Solanum dulcamara*, but were not detected in any other species.

Xanthophylls

The distribution of the common xanthophylls in the berries examined is given in Table 6. Apart from *Cotoneaster hebephylla* (discussed below) a characteristic feature is the complete absence of new pigments.

Cotoneaster hebephylla. Two pigments present in traces occurred adsorbed between aurochrome and flavoxanthin. These pigments have absorption spectra identical with those of flavoxanthin and aurochrome respectively. From their adsorptive properties they are probably monohydroxy derivatives of the two parent pigments.

Rhodoxanthin. The absorption spectra in two solvents obtained with chromatographically pure rhodoxanthin obtained from the arils of *Taxus baccata* are given in Fig. 2. It will be seen that although the main maxima correspond well with

those of Kuhn & Brockmann (1933) (500 and 502 mμ. in benzene and 484 and 487 mμ. in light petroleum) there was little sign of the two subsidiary bands reported by Kuhn & Brockmann. As it is known now that ketocarotenoids rarely have more than one absorption band (Goodwin, 1952*a*), it is possible that the earlier observations with less accurate spectroscopic instruments were incorrect in detail. Two compounds closely related to rhodoxanthin, but with absorption maxima at slightly lower wavelengths, were also observed in our extracts; they are probably *cis* isomers of rhodoxanthin. Lubimenko (1920) also noted a number of different modifications of rhodoxanthin.

DISCUSSION

As long ago as 1914 Lubimenko (1914) noted that lycopene was present in *Arum italicum* only in small amounts, together with large amounts of a '*Lykopin erster Ordnung*'; this Kylin (1927) observed in *A. italicum* and *A. orientale* and named arumin. *A. maculatum* has been examined only once previously when lycopene was isolated (Karrer & Wherli, 1930); the presence of other pigments was not mentioned. It is now obvious that '*Lykopin erster Ordnung*' and arumin are mixtures of prolycopenes. 'Arumin' and '*Lykopin erster Ordnung*' were also noted in *Rosa canina* and *Solanum dulcamara* (Lubimenko, 1920; Kylin, 1927); in the present work prolycopenes have also been identified in these species.

It is difficult to identify Kylin's sorbusin, which he found in his specimens of *S. aucuparia*, with any pigments observed in the present investigation.

Capsicum annuum and *Rosa* spp. have been studied many times previously (see e.g. Goodwin, 1952*a*), and the only novel feature of the present investigation is the detection for the first time of phytoene in *C. annuum*.

The *Cotoneaster* spp. examined here have not previously been investigated, although lutein and violaxanthin, but not β-carotene, were detected in traces in *C. occidentalis* (Karrer & Rutschmann, 1945); 'lipochromes' were reported in an unnamed *Cotoneaster* sp. by Kohl (1902). Similarly, the only previous report of carotenoids in *Crataegus* spp. is of 'lipochromes' in *C. crusgalli* (Thudichum, 1869).

Hippophaë rhamnoides has been previously investigated by Karrer & Wherli (1930), who reported the presence of traces of zeaxanthin. *Lonicera periclymenum* closely resembles the related *L. japonica* (Goodwin, 1953*a*), differing only in producing less phytofluene and no η-carotene.

Pyracantha rogersiana berries are similar to *P. coccinea* (Karrer & Rutschmann, 1945) in producing only traces of carotenoids. *P. flava*, on the other hand, synthesizes considerably more, with the hydrocarbon fraction (about 50% of the total pigments) consisting exclusively of β-carotene with a minute trace of phytofluene. This is again in contrast to another species, *P. angustifolia*, in which, although β-carotene remains the main hydrocarbon component, large amounts of prolycopenes I–VI and phytofluene are found (Zechmeister & Sandoval, 1945). Further evidence that there is considerable inter-species variation in the genus *Pyracantha* comes from the observation that *P. yunanensis* produces much less phytofluene (0·04 mg./kg. fresh wt.) than does *P. angustifolia* (15–28 mg./kg. fresh wt.) (Zechmeister & Sandoval, 1945).

Lycopene has been confirmed as the major hydrocarbon of *Solanum dulcamara* (Zechmeister & Cholnoky, 1936) and *Tamus communis*; phytofluene, phytoene and β-carotene were, however, observed for the first time in *T. communis*. The presence of lycoxanthin and lycophyll was also confirmed in *S. dulcamara*, but, owing to the very low percentage of xanthophylls in our specimens of *T. communis*, and the small amounts of berries available, it was not possible to look for them in this species.

The presence of rhodoxanthin in the arils of *Taxus baccata* has been confirmed (Kuhn & Brockmann, 1933), and a number of *cis* isomers also appeared on the column; it is not known whether these are artifacts or not, but would explain Lubimenko's (1914) observation of five different modifications of rhodoxanthin in his specimens. The hydrocarbons in *T. baccata*, in which β-carotene predominates, have not been previously described.

General conclusions

The following general conclusions can be drawn from these and previous investigations on berries (see Goodwin, 1952*a*). (*a*) β-Carotene is probably always present in berries to some extent; the failure to record β-carotene in some previous experiments (see Goodwin, 1952*a*, pp. 36–37) is almost always associated with the presence of lycopene; when lycopene is present β-carotene can often be a minor constituent (Table 5) and this may well have been missed or ignored in earlier work. (*b*) Lycopene, often considered a characteristic polyene of fruit, is often absent, but when it is present one or more members of the phytofluene series are also present, although tetrahydrolycopene (neurosporene) is conspicuous by its absence. (*c*), When β-carotene is the only hydrocarbon present, it is accompanied by the xanthophylls characteristic of green leaves and also by chlorophyll; in these cases the total carotenoid content of the berries is rather low. (*d*) Chlorophyll is not detectable in fully ripened lycopene-containing berries. (*e*) Crypto-

xanthin is almost always present. (*f*) Lutein, the main carotenoid of green leaves, is often absent.

The present investigation further emphasizes the view (Goodwin, 1952*a*) that the distribution of carotenoids in berries appears at the present time to have no obvious taxonomic significance. The best example in support of this is the great species differences observed in the genus *Pyracantha* (discussed above). The situation is so complex that many more surveys of the present type will be necessary to reveal possible taxonomic correlations.

The failure to observe tetrahydrolycopene (neurosporene) along with the other members of the Porter–Lincoln series in most lycopene-containing berries further suggests that this series is synthesized in parallel fashion rather than sequentially (Goodwin, 1953*b*).

SUMMARY

The complete polyene distribution has been determined for berries of twenty-three species representing eight families.

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